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COLORACIÓN BASADA EN CAROTENOIDES EN *Lacerta vivipara*, JACQUIN 1787

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IMPLICACIONES AMBIENTALES, FISIOLÓGICAS Y POBLACIONALES

TESIS DOCTORAL

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Museo Nacional de Ciencias Naturales

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Implicaciones ambientales, fisiológicas y poblacionales

Memoria presentada por **Luis M. San José García** para
optar al Grado de Doctor

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A Sergio

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Presentación

Los carotenoides son unos pigmentos que solamente son sintetizados por plantas, algas, y algunas bacterias y hongos. Los animales, por tanto, los obtienen a través de la dieta, utilizándolos posteriormente para distintas funciones. Entre éstas, los carotenoides son ampliamente conocidos por producir ornamentos muy llamativos por sus distintos tonos de amarillo, naranja y rojo y que median, como señales, las interacciones entre individuos de una misma especie o entre individuos de distintas especies. Debido a que los animales no pueden sintetizar carotenoides, no pueden producir los ornamentos de forma ilimitada, viéndose restringidos en cuántos carotenoides pueden invertir para producir ornamentos más vistosos que les permitan, por ejemplo, ser más atractivos, más amenazantes, o que les otorguen un mayor status social. Más aún, la función antioxidante e inmunoestimuladora de los carotenoides, obliga a los animales a invertir los carotenoides en funciones ornamentales según las restricciones que imponga el mantenimiento de su propia salud. De esta forma, la expresión de ornamentos basados en carotenoides se convierte en una función directa de la calidad de los individuos que los portan, ya que éstos producirán mejores y más vistosos ornamentos cuanto más capaces sean de obtener carotenoides del medio y cuanto mejor sea su condición y salud. Consecuentemente, se ha observado que los ornamentos basados en carotenoides funcionan como señales honestas de la condición de los individuos, siendo evolutivamente estables por los beneficios que surgen tanto para el emisor como el receptor del hecho de producir y responder, respectivamente, a estas señales.

Sin embargo, el conocimiento que actualmente existe entorno a la producción de ornamentos basados en carotenoides así como entorno a los mecanismos que guían su evolución está muy restringido a peces y, especialmente, a aves. A través de estudios en estos grupos de animales se han alcanzado la serie de paradigmas expuestos arriba cuya generalidad, sin embargo, no se ha testado en otros grupos de animales. Las lagartijas, al igual que peces y aves, presentan coloraciones muy vistosas que tienen una función determinante en las interacciones intraespecíficas. Sin embargo, el papel que juegan los carotenoides en estas coloraciones y si dicho papel cumple con los paradigmas establecidos a partir de los profusos estudios hechos en aves y peces, continua siendo un misterio.

El objetivo de la presente tesis doctoral es testar la generalidad de dichos paradigmas, investigando el papel que los carotenoides pueden tener en la coloración ventral de la lagartija de turbera, *Lacerta vivipara*. Estudios preliminares sugieren que la coloración ventral de esta especie podría ser un ornamento que indique la condición de los individuos mientras que, por el contrario, otros estudios sugieren que la coloración es un carácter polimórfico que varia de forma discreta y bajo un estricto control genético. Por lo tanto, estos contradictorios estudios sugieren que los carotenoides podrían ser los responsa-

bles de, o bien coloraciones dependientes de la condición y, por lo tanto, en consonancia con los paradigmas establecidos en aves y peces o, por el contrario, dar lugar a un polimorfismo de marcado carácter genético, contradiciendo entonces dichos paradigmas. Por lo tanto, esta especie ofrece como modelo animal la oportunidad idónea para ampliar el conocimiento entorno a los ornamentos basados en carotenoides, permitiendo apoyar o rechazar la generalidad de los paradigmas obtenidos mediante estudios en aves y peces.

Chapter I

General Introduction

Carotenoid pigments account for many of the most overwhelming animal ornaments, colouring animal integument with different tones of yellow, orange, and red. Examples of carotenoid-based ornaments exist all along the tree of life including birds (Hill and McGraw 2006), fish (Endler 1983), reptiles (Macedonia et al. 2000), amphibians (Matsui et al. 2002; Richardson et al. 2009), insects (Bezzerides et al. 2007; Sandre et al. 2007; Moran and Jarvik 2010), crustaceans (Wade et al. 2009), and many other invertebrate groups (*e.g.*, acanthocephalans; Kaldonski et al. 2009, starfishes; Maoka et al. 2010, urochordata; Hirose et al. 1998). During the last three decades, carotenoid-based ornamentation has become one of the most studied traits in the field of animal signalling. Although the presence of carotenoids in animal ornaments was known for decades (*e.g.*, Lonnberg 1938; Kritzler 1943; Fox et al. 1967), it was during the 80's and early 90's when a series of influential papers set the foundations for the study of carotenoid-based ornaments in a visual-signalling context. Endler's pioneering work on Trinidadian guppies, *Poecilia reticulata*, provided the first clear demonstration that **i.** females actively select males with more intense carotenoid-based ornaments, and **ii.** males displaying more intense carotenoid-based ornaments incur in more costs (higher predation) than those showing less intense ornaments (Endler 1980; Endler 1983). With his studies, Endler underlined the relevance of natural and sexual selection for the evolution of carotenoid-based signals. Just a few years later, Kodric-Brown (1985) suggested that male carotenoid-based coloration functions as an honest signal of phenotypic and genetic quality, which was later supported by studies showing positive relationships of individual quality and condition with ornament intensity and male mating success in fish and birds (Kodric-Brown 1989; Hill 1990; Milinski and Bakker 1990; Hill 1991; Burley et al. 1992; Hill 1992; Hill 1994b). Since these studies, the study of carotenoid-based signals became popular and provided one of the best evidences for condition-dependent signalling in animals and for the general mechanisms assuring honesty and evolutionary stability of signals (Griffith et al. 2006).

Carotenoids and Colour Production

The term carotenoid includes more than 750 different fat-soluble hydrocarbons that are synthesized by plants, algae, and some bacteria and fungi (Goodwin 1986). Animals cannot synthesize carotenoids *de novo* (but see Moran and Jarvik 2010 for a remarkable exception) and must obtain them with the diet (Goodwin 1986). The common structure of carotenoids is a C_{40} isoprenoid with none, one, or two terminal carbon rings (Britton 1995). Carotenoids are classified as carotenes when formed only by carbon and hydrogen atoms and as xanthophylls when oxygen is also present in the molecule (Fig. 1a; Britton 1995). Carotenoids present a conjugated structure, *i.e.* a system of alternating single and double bonds where electrons are delocalized (Britton 1995). This structure is responsible for the light absorbing properties of carotenoids (Britton 1995). Because electrons in the conjugated system are highly delocalized, light of relatively low energy is enough to promote the transition of electrons to an excited state. Carotenoids therefore

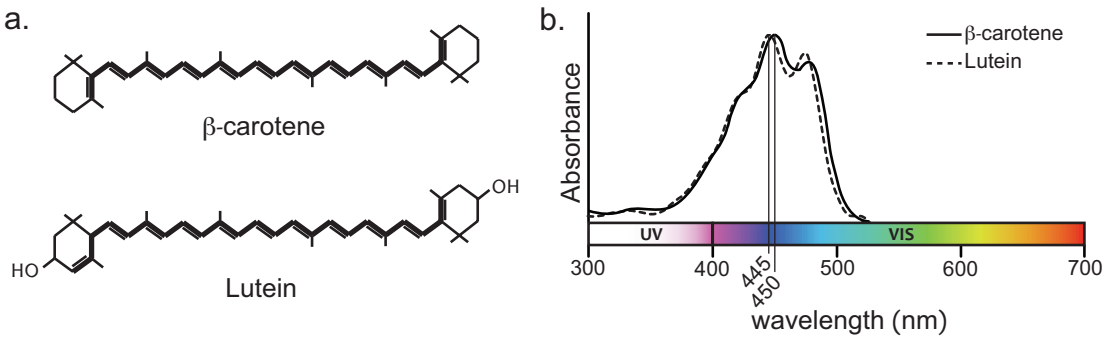

FIG. 1.—a. Chemical structure of a carotene (β-carotene) and a xanthophyl (lutein). The conjugated double-bond system responsible of colour is highlighted in bold. **b.** Ultraviolet (UV) and visible (VIS) light absorption spectra of β-carotene and lutein. The distinct conjugated structure of the two carotenoids translates into differences in the spectral location of the maximum absorbance peak.

absorb short wavelength light, mainly between 400 to 500 nm (Fig. 1b). Differences between carotenoids in their conjugated structure, for instance, in the number of double bonds, leads to differences in some of their absorbing properties (*e.g.,* maximum wavelength absorbance; Fig. 1b).

However, carotenoids cannot colour animal integument exclusively by themselves. Carotenoids have pure pigment effect and do not emit light in the visible or UV range. In order to produce colour, carotenoids always require the presence of other integumentary components that reflect light (Shawkey and Hill 2005). Such components reflect the light previously filtered by carotenoids (Fig. 2). Because carotenoids absorb visible light of short wavelength (violet to blue), less short-wavelength light is reflected, leading to the predominance of longer wavelengths and, hence, to the common yellow, orange, and red appearance of carotenoid-based ornaments (Fig. 2). As an additional consequence, carotenoid deposition in the integument can produce an ultraviolet (UV) peak given that carotenoids increase the contribution of UV reflectance in relation to violet-blue reflectance (Fig. 2). The effect of carotenoid deposition on coloration depends on the reflective properties of the integument. For instance, carotenoid deposition will produce no UV peak if the integument does not reflect light in the UV range. Similarly, if the integument barely reflects visible long-wavelength light, carotenoid deposition cannot produce yellow, orange, or red ornaments (this latter situation explains the existence of blue to green carotenoid-based ornaments of some species; *e.g.*, blue-footed booby, *Sula nebouxii*; Velando et al. 2006).

Intraspecific colour variation resulting from differential integumentary deposition of carotenoids is relatively well understood (see for example Andersson and Prager 2006). Carotenoids mainly affect the chromatic component of coloration, because, as explained above, they affect the relative contribution of short and long wavelength light (Jacot et al. 2010). Increased carotenoid deposition in the integument decreases reflectance at violet to blue wavelengths, which translates into increased saturation and decreased brightness of carotenoid-based ornaments (see Box 1 for definitions of colour parameters). Changes in carotenoid composition commonly lead to changes

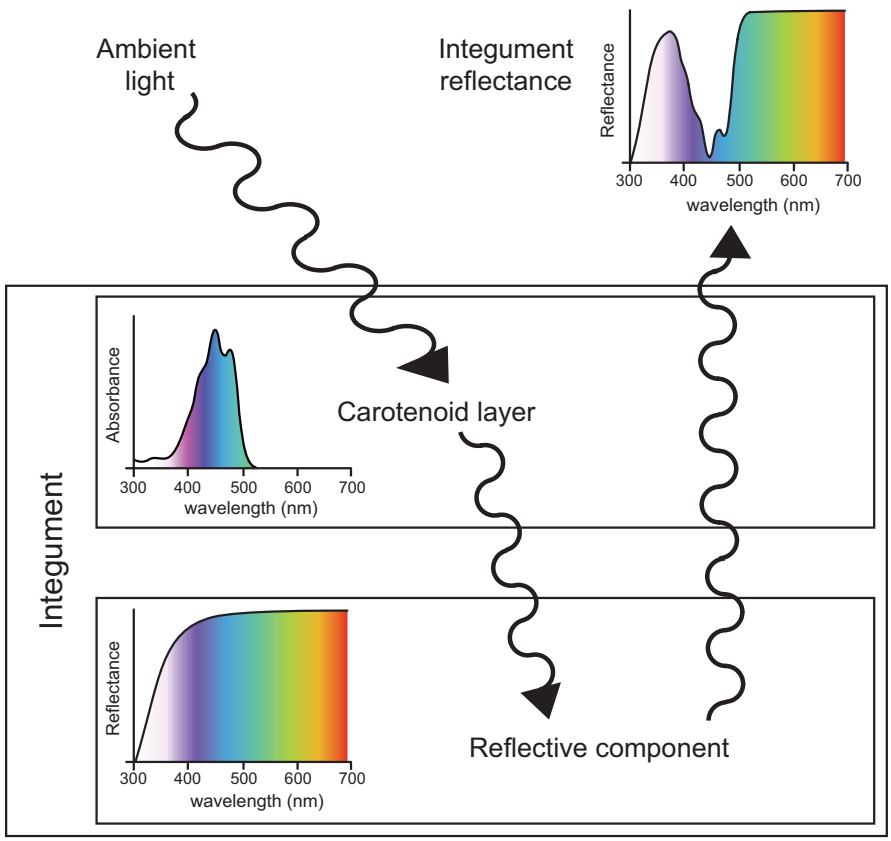

FIG. 2.—Basic scheme of colour production in carotenoid-based ornaments. Ambient light reaches the integument and encounters carotenoids, which predominantly absorb wavelengths between 400 and 500 nm. Not absorbed light then reaches the reflective component of the integument. In the example, the reflective component reflects light with similar intensity at almost all wavelengths but, because carotenoids have previously subtracted short visible wavelengths, it predominantly reflects long-wavelength (above 500 nm) and UV light.

in hue values, given that different carotenoid species have different maximum absorbance wavelengths.

In contrast, colour variation resulting from other integumentary components present in carotenoid-based ornaments is hardly known. Carotenoid-based colorations can be the consequence of very complex integumentary designs (Table 1). Carotenoids can occur in the integument together with more than one kind of reflective structure and with more than one type of pigment. These components can contribute to interspecific colour variation and their colour effects could be even confounded with those usually attributed to carotenoids. To date, only few studies have considered their potential effects on carotenoid-based ornaments (Grether et al. 2001; Grether et al. 2004; Shawkey et al. 2006; Jacot et al. 2010) and important questions still remain unsolved.

Table 1.—Summary of the different integumentary components present in carotenoid-based ornaments of vertebrates. In birds, keratin-based structures of feathers and collagen-based structures of bare parts provide carotenoids with a reflective background. In other taxa, iridophores, which contain purine crystals, and the basal collagen layer of the dermis function as reflective components. A special type of iridophores, leucophores, is common in the fish integument (for more details see; Menter et al. 1979). In birds, no other yellow-red pigments besides carotenoids are present in the integument whereas pteridines, which also absorb blue to green light, are commonly found in the integument of fish, amphibians, and reptiles. Finally, melanin pigments can also occur in some carotenoid-based ornaments.

Taxa	Reflective components	Yellow-red pigments	Other pigments	References
Birds				
Plumage	keratin	carotenoids	melanins	(Hill and McGraw 2006)
Bare parts	collagen	carotenoids	melanins	(Hill and McGraw 2006)
Fish	iridophores, collagen	carotenoids, pteridines	melanins	(Fujii 1993)
Amphibiana	iridophores, collagen	carotenoids, pteridines	melanins	(Zug et al. 2001)
Reptiles	iridophores, collagen	carotenoids, pteridines	melanins	(Cooper and Greenberg 1992)

Carotenoid-based Signalling

Animal Signals and Signal Honesty

Defining animal signalling and communication is one of the most intricate questions in behavioural and evolutionary ecology (see some recent reviews; Hurd and Enquist 2005; Scott-Phillips 2008; Carazo and Font 2010; Owren et al. 2010; Scott-Phillips et al. *in press*). Obtaining a robust definition of what is and what is not a signal, signalling, or communication in animals is complicated because each proposed definition always includes or excludes some cases or examples whose consideration is controversial (Bradbury and Vehrencamp 1998). Thus, many different definitions have been proposed (*e.g.*; Wilson 1975; Enquist 1985; Hasson 1994; Hasson 1997) but none has been widely accepted. Maynard-Smith and Harper (1995) provided a definition of animal signals, which is quite precise, although not being exempt of some controversy (see for instance Stenseth and Sætre 2004; Carazo and Font 2010). They define a signal as "any act or structure which alters the behaviour of other organisms, which evolved because of that effect, and which is effective because the receiver's response has also evolved" (Maynard-Smith and Harper 1995; Maynard-Smith and Harper 2003). The relationship between the signal and the receiver's response constitute 'signalling' (Wilson 1975), and only when signalling is successfully completed; *i.e.*, the signal is received and elicits a response on the receiver, we should talk about 'communication' (Scott-Phillips 2008).

Maynard-Smith and Harper's definition (2003) emphasizes the (co)evolutionary mecha-

Box 1. Colour quantification

Hue, chroma, and brightness define a three-dimensional space known as the tristimulus colour space. As defined by Endler (Endler 1990), "[h]ue is the everyday meaning of 'colour', e.g. violet, blue, green, yellow, orange, red". Each hue value is characterized by a particular value of chroma, which measures the degree of colour saturation or purity, and brightness, which measures the intensity (Fig. I). There exist different methods to objectively measure hue, chroma, and brightness (review in Montgomerie 2006). Reflectance spectrometers have become one the most common equipment for measuring colouration in ecological and evolutionary studies. Depending on their configuration and the used light source, they can measure the complete reflectance spectrum of the animal integument, including the UV and visible range. Reflectance is measured as the ratio between reflected and incident light for a given surface and angle of incident light. Reflectance spectrometers allow measuring reflectance under standard conditions of incident light, which thereby allow comparing colour among and within individuals.

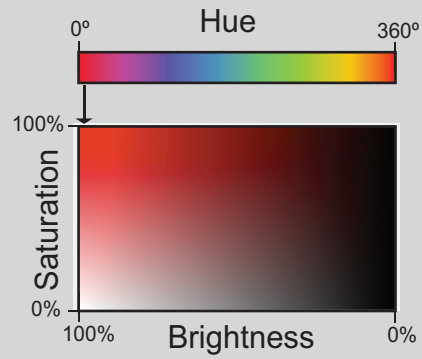

Fig. I.—Colour classification in a tristimulus colour space.

Reflectance data can be used to derive different measures of hue, chroma, and saturation (Endler 1990; Montgomerie 2006; Butler et al. 2011). Hue and chroma measure the chromatic component of the spectrum, which is a function of the shape of the spectral curve. Hue is related with the wavelength of the maximum slope as well as with the sign of this slope. Hue decreases as maximum wavelength shift to longer wavelengths, which results in redder colours (Fig. IIa). Chroma is a measure of the relative contribution of the reflectance of different spectral intervals to the overall reflectance. Thus, chroma increases and colour becomes more saturated or purer as the spectral curve is more predominantly formed by a single and narrow spectral interval (Fig. IIb). Brightness measures total reflectance, increasing with the height of the spectral curve (Fig. IIc). It accounts for the achromatic component of the spectrum because it does not depend on spectral shape.

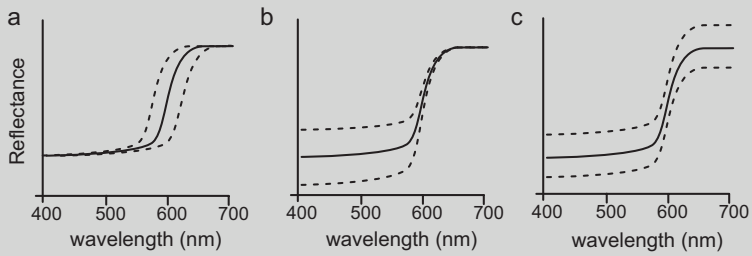

Fig. II.—Reflectance spectra with different values of hue (**a**), chroma (**b**), and brightness (**c**).

nisms that lead to animal signalling. It implicitly states that signals evolve because of their fitness effects on two interacting parts; the signaller and the receiver. Signallers benefit from signalling because the behavioural response that signals elicit on receivers is, on average, advantageous for them. No signal is expected to evolve if signallers obtain no benefit from signalling either because they elicit no response or because the receivers' response results in no fitness gain. Similarly, signals elicit receivers' response because such response is, on average, advantageous for receivers. If (on average) receivers do not benefit of responding to signallers, they are expected to ignore them, which finally ends the signalling system. In summary, the evolutionary stability of signalling systems is predicted to occur only when, on average, signallers and receivers optimize their fitness by, respectively, signalling and responding.

A signalling system may still be evolutionary stable even if it occasionally results in no fitness gain for either signallers or receivers. For instance, producing a signal or responding to it may reduce fitness by increasing predation risk and there may exist signal perception and/or interpretation errors resulting in receivers' responses that are not appropriate and hence, that do not produce the expected benefits. As long as fitness gains derived from signalling surpass potential fitness losses, the signalling system will prevail, evolving to minimize such fitness losses. A more relevant threat for the stability of most signalling systems is that some signallers may also increase their fitness by cheating. Cheaters elicit appropriate behavioural responses on receivers, which, however, do not obtain the benefits expected from responding to the signal. Thus, in order to be evolutionary stable most signalling systems require mechanisms that ensure signal honesty and prevent cheating given that, otherwise, receivers would cease to respond to signals because of the costs arising from responding to cheaters.

How signal honesty originates and, hence, how signalling systems are evolutionary stable has become one of the most interesting debates in behavioural and evolutionary sciences (Wilson 1975; Zahavi 1975; Dawkins and Krebs 1978; Hamilton and Zuk 1982; Enquist and Leimar 1983; Grafen 1990; Guilford and Dawkins 1991; Hasson 1991; Endler 1992; Pomiankowski and Iwasa 1993; Johnstone 1995; Maynard-Smith and Harper 1995; Endler 2005). This debate has resulted in different hypothesis; some of these are summarized below.

The handicap principle.—Some traits evolve as honest signals because their development, maintenance, and/or display entails costs that may compromise survival or future reproduction. Signal expression is thus related with individual quality to sustain such costs and, hence, signals may function as indicators of such quality. Cheating is prevented because signal-derived costs surpass the benefits that individuals without such quality would receive from producing a fake signal and posing as having such quality. This idea was first presented by Zahavi (1975) under the name of the 'handicap principle'. Initially, the handicap principle was highly criticized, given that mathematical models did not fit the predictions of Zahavi's verbal model (see for instance; Maynard-Smith 1976). This conflict originated because Zahavi's model predicted signal costs to increase with the expression of the ornament used as handicap: "[…] the more developed the character the more severe was the test" (Zahavi 1975). However, different models indicate that signal costs have to be differentially scaled among individuals of different quality in order to

evolve as predicted by the handicap principle (Bell 1978; Enquist 1985; Pomiankowski 1987; Grafen 1990; Maynard-Smith 1991, although see Getty 1998; Hausken and Hirshleifer 2004; Számadó 2011, for alternative models and interpretations). Costly signals are thus likely to be evolutionary stable if signal costs increase with decreasing quality (see Fig. 3a). Therefore, only high-quality individuals gain a net benefit from signalling, given that they pay relatively lower costs of signalling. Contrary, low-quality individuals gain no net benefit from signalling (*i.e.*, of cheating) since they pay relatively higher costs of signalling (see Fig. 3a). The handicap principle also applies to honest signals of 'need' (*i.e.*, signals used by individuals differing in their requirements for a particular resource; Johnstone 1997). In these cases, costly signals are likely to be evolutionary stable when costs increase with signal expression, but, in contrast with signals of 'quality', honesty is maintained because benefits are differentially scaled among individuals differing in need (higher for high-need individuals than for low-need individuals, see Fig. 3b). Maynard-Smith and Harper (2003) suggested that the general condition for the handicap principle is that "the ratio of the cost of a signal to the benefit received is lower for individuals giving stronger signals" (see also Getty 1998).

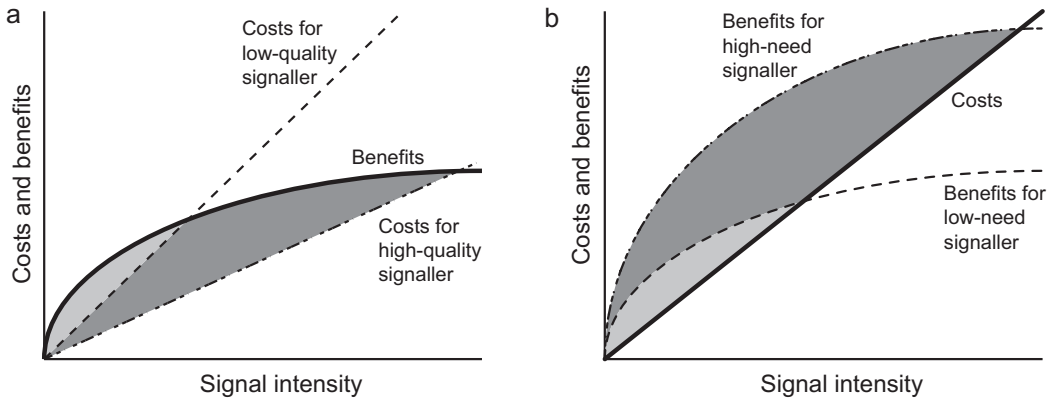

FIG. 3.—Relationship between signal intensity and costs and benefits with respect to signal intensity predicted under the handicap principle. Shading indicates the parameter space with net benefits. **a**. In quality-based signals, costs associated with the signals (doted lines) increase faster in low-quality than in high-quality signaller, and benefits linearly increase with signal intensity (solid line). Thus, only high-quality individuals gain a net benefit from producing very intense signals (dark+light-shaded areas), while in low-quality signallers the benefits are only present when producing low intensity signals (light-shaded area). **b**. In signals of need, it is predicted that benefits (doted lines) increases faster with need, and costs (solid line) increase with increasing signal intensity. Thus, only individuals in higher need gain a large net benefit when producing a high intensity signal (dark+light shaded area), while low-need signallers only gain net benefits when producing low intensity signals (light-shaded area). Modified from Johnstone 1997.

Social punishment.—Social punishment may also assure signal honesty (Møller 1987; Lachmann et al. 2001). A signal may honestly reflect a certain quality or condition if such quality is usually challenged and verified during continuous signaller-receivers encounters (Lachmann et al. 2001; Számadó 2011). For instance, male house sparrows, *Passer domesticus*, exhibit black

'bibs' that serve as signals of their resource-holding potential (Møller 1987). Approaching-retreating interactions and eventual fights guarantee that only good quality individuals can exhibit intense signals, since it prevents individuals from cheating (Møller 1987). The frontier between social punishment and a handicap for display is not really clear (Számadó 2011), given that social punishment can also be envisaged as a cost; a cost that directly applies to cheaters instead of a cost that applies to all signallers; *i.e.*, to potential cheaters as well as to honest signallers (Hurd 1995; Hurd and Enquist 2005). Given the differences with the exact formulation of the handicap principle, some authors consider signalling systems that penalize cheaters as an alternative explanation to the problem of honesty (Számadó 2003) whereas other authors consider them merely as a deviation of the handicap principle ('interaction handicap'; Hurd and Enquist 2005).

Indices.—Honesty can be also prevented when the signal is physically associated with the quality or condition that it directly reflects (Maynard-Smith and Harper 1995; Vanhooydonck et al. 2007). These kinds of signals are referred as indices.

Shared interest.—When signaller and receiver share a common interest, *i.e.*, place the possible outcomes of the signalling interaction in the same order of preference, cheating will be a suboptimal strategy even for a cost-free signal (Maynard-Smith 1991; Maynard-Smith 1994). For instance, colourful traits used for species recognition are suggested to be honest because both signaller and receiver benefit from avoiding mating with an individual of a closely related species (Alatalo et al. 1994).

Loss of inclusive fitness.—When signallers and receivers are related, benefits that may be obtaining by cheating may translate into loss of inclusive fitness (Johnstone and Grafen 1992). Signals used by social insects are usually suggested to be honest because of this mechanism.

Arbitrary signals.—Male ornamental traits that signal no additional information than its potential to correspond to receiver preference (attractiveness) are considered as arbitrary signals or Fisherian traits (Candolin 2003; Prum 2010). Arbitrary signals are neither honest nor dishonest, because receiver benefits merely arise from the inheritance of signal expression *per se* (*i.e.*, of attractiveness) and not from the inheritance of any signaller quality related with attractiveness that could be falsified (Fisher 1930; Kokko et al. 2003).

Condition-dependent Signalling through Carotenoid-based Ornaments

Because of their particular biological properties (*i.e.*, antioxidant capacity, immune enhancing effects, etc.), carotenoids seem to have a great potential to serve as honest indicators of quality and condition (Gray 1996; Badyaev and Hill 2000). Carotenoid-based ornaments become honest indicators of quality because carotenoids are causally related with phenotypic and genetic quality, mainly with nutritional and health status of individuals (Hamilton and Zuk 1982; Kodric-Brown and Brown 1984). In this sense, carotenoid-based signals cannot be bluffed because low quality individuals cannot assume the physiological costs and environmental constraints that results from the development, maintenance, and display of ornaments. Thus,

carotenoid-based ornaments are commonly suggested to evolve as handicaps (*sensu* Zahavi 1975) although, in some cases, they may also function as signals of social status and, hence, subjected to social punishment, or as indices (Maynard-Smith and Harper 2003). Below, we present the distinct mechanism proposed by which carotenoid-based ornaments may be honest indicators of quality.

Constraints on the Development and Maintenance of Carotenoid-based Ornaments

Carotenoids are limited and difficult to obtain—Animals cannot synthesize carotenoids and, hence, development and maintenance of carotenoid-based ornaments may be limited by individual capacity to obtain carotenoids from the diet (Badyaev and Hill 2000). The fact that dietary carotenoids may constrain the expression of carotenoid-based ornaments was known for long time in poultry science (review in Brush and Power 1976), but first experimental evidences relating dietary carotenoids with visual signalling were provided at the end of the 80's. Kodric-Brown (1989) working on Trinidadian guppies and Hill (1992) working on house finches demonstrated that dietary supplementation of carotenoids improves the expression of carotenoid-based colorations. These studies have been replicated in more bird and fish species with similar results (Hill and Benkman 1995; Evans and Norris 1996; McGraw et al. 2001; McGraw et al. 2002b; Navara and Hill 2003; Kalinowski et al. 2005; Clotfelter et al. 2007; Costantini et al. 2007; Baron et al. 2008; Pike et al. 2010; Benito et al. 2011), supporting that the capacity to produce more intense carotenoid-based ornaments is generally limited by the amount of dietary carotenoids and, hence, by individual capacity to obtain carotenoid rich diets. Carotenoid-based ornaments may thus signal qualities like foraging ability, territory quality, or social status, which control food and, hence, carotenoid access (Badyaev and Hill 2000). Environmental scarcity of carotenoids prevents cheating given that low quality individuals cannot obtain the necessary carotenoids.

The publication of the studies of Kodric-Brown (1989) and Hill (1992) was accompanied by an intense debate about whether animals are really limited in natural systems (Hill 1994a; Hudon 1994; Thompson et al. 1997). Although, nowadays, it is generally assumed that carotenoids are scarce in the wild, still few evidences have been obtained from studies in natural conditions (Pérez-Rodríguez 2009; Prum 2010; Svensson and Wong 2011). Geographical (*i.e.*, among populations) and temporal variation of carotenoid availability has been observed (Slagsvold and Lifjeld 1985; Hill 1993b; Hill 1993a; Ryan et al. 1994; Linville and Breitwisch 1997; Isaksson and Andersson 2007; Sillanpää et al. 2008; Eeva et al. 2010) but only one study conducted in Trinidadian guppies showed a clear relationship among geographical variation in carotenoid availability, carotenoid intake, and intensity of carotenoid-based ornaments (Grether et al. 1999) although see (Schwartz and Hendry 2010). However, the fact that carotenoid abundance may vary annually or among population may not explain why carotenoid availability may differ among individuals within the same population, which is the level at which selective pressures for honest signalling are acting. Unfortunately, studies considering carotenoid availability at the population level are less abundant. Hill et al. (2002) measured gut carotenoid content in wild

house finches and found that the more carotenoids in the gut the redder coloration was. However, this finding was not consistent across different populations (Hill et al. 2002). Moreover, they also found that the more carotenoids in the gut the brighter coloration was, which contradicts the expected effects of increased carotenoid deposition in the integument and suggests that the relationship between gut carotenoids and coloration was not exclusively mediated by integumentary carotenoids (Jacot et al. 2010). To date, the best examples of dietary carotenoid limitation in the wild have been provided by several experiments conducted in nestling birds and colonial nesting birds, where the access to dietary carotenoids is relatively easy to manipulate (Fitze et al. 2003b; Tschirren et al. 2003; Biard et al. 2006; Casagrande et al. 2007; Ewen et al. 2008; Thorogood et al. 2008; Morales et al. 2009). In great tits, *Parus major*, Fitze et al. (2003b) experimentally showed that carotenoid-supplemented nestlings developed more intense yellow plumage colorations than nestlings fed with a placebo containing no carotenoids, supporting that the development of nestling coloration is constrained by parental food provisioning in the wild (Fitze et al. 2003a). In conclusion and in contrast with evidences from laboratory studies, clear experimental evidences on the dietary-dependency of carotenoid based ornaments in wild systems are still weak and best demonstrations of this paradigm are limited to a few bird species and model systems.

It has also been suggested that the physiological mechanism leading to absorption, transport, and deposition of carotenoids may constrain the development and maintenance of carotenoid-based ornaments (Grether et al. 1999). Carotenoid absorption seems to be a highly inefficient process given that animals uptake only a small part of the carotenoids they ingest (Furr and Clark 1997). Some authors suggest that carotenoid absorption is an energy demanding process that could be limited by the energetic resources of animals (Hill 2000). However, this hypothesis seems unlikely given that carotenoids are absorbed by passive diffusion (Furr and Clark 1997) and that the specific membrane transport proteins observed in some species seem to function without energy expenditure (Yonekura and Nagao 2007). Carotenoid absorption may be also limited by the amount of dietary lipids in the diet, given that lipids provide a hydrophobic domain that carotenoids require for their absorption (Erdman et al. 1993; van het Hof et al. 2000). Low dietary content of lipids has been suggested to limit carotenoid absorption and, thereby, the expression of carotenoid-based ornaments (Hill 2000; McGraw and Parker 2006; Fitze et al. 2007). However, the relevance of dietary lipids for carotenoid absorption remains hardly known in species with carotenoid-based ornaments and most of the current knowledge comes from studies in humans and other mammals (Parker 1996).

Once absorbed, carotenoids are specifically transported with other lipophilic molecules by chylomicrons and lipoproteins (Erdman et al. 1993; Parker 1996). McGraw and Parker (2006) showed that an experimental increase of lipoprotein levels positively affected carotenoid-based coloration of zebra finches, *Taeniopygia guttata* (McGraw and Parker 2006). In a second study, McGraw et al. (2006a) showed that lipoprotein levels increase with increasing circulating testosterone levels, suggesting that carotenoid transport and, hence, coloration is a function of an individuals' capacity to deal with the detrimental effects of testosterone (Peters 2007). Carotenoid

transport also depends on dietary lipids given that they stimulate the formation of chylomicrons, which are thereafter assembled into lipoproteins (Borel et al. 1998; Silva et al. 2003). In fact, McGraw and Parker (2006) enhanced lipoprotein status and coloration of zebra finches through the dietary administration of cholesterol, which supports that lipoprotein levels may be also environmentally determined by dietary lipid intake. However, cholesterol is not a naturally ingested lipid and, hence, additional studies are needed to fully test this hypothesis.

Some species preferentially deposit in their ornaments carotenoids that are not directly obtained with the diet but metabolized from other dietary carotenoids (Hata and Hata 1972; Brush 1990; Ohkubo et al. 1999; McGraw et al. 2001; McGraw et al. 2002a; McGraw 2004; McGraw et al. 2006d). Metabolized carotenoids usually account for redder colorations because they absorb light of longer wavelength than carotenoids used as metabolic substrates (McGraw et al. 2003; Prager et al. 2009). Therefore, it has been suggested that in order to produce more attractive colorations, individuals may expend more energy in metabolic routes that produce redder carotenoids, which only high-quality individuals might afford without detrimental consequences for other physiological functions (Hill 2000; McGraw et al. 2005). Hill (2000) specifically tested this hypothesis in house finches, *Carpodacus mexicanus*, which present a red plumage that results from the metabolism of yellow dietary carotenoids like β-cryptoxanthin. He demonstrated that food restriction in birds supplemented with β-cryptoxanthin prevents the appearance of redder plumages, which suggests that carotenoid metabolism is restricted in individuals with low energy levels.

Carotenoids are demanded for health maintenance—Hamilton and Zuk (1982) presented a model for the evolution of ornaments as indicators of individual health condition. Hamilton and Zuk's model states that female mate choice based on epigamic traits might evolve if **i.** the expression of such traits reflects individual health, **ii.** diseases affect health and thereby coloration, and **iii.** disease resistance has an heritable basis allowing that females benefit (via "good genes") when mating with healthier and, hence, more ornamented males. After Hamilton and Zuk published their model, several studies successfully demonstrated that epigamic traits including carotenoid-based ornaments reflect health status (Milinski and Bakker 1990; Zuk et al. 1990; Houde and Torio 1992). However, still an important question remained unsolved in relation to how carotenoid-based ornaments are related to health (Folstad and Karter 1992). Lozano (1994; see also; Shykoff and Widmer 1996) provided an explanation for this question. Based on epidemiologic, nutritional, and immunological studies conducted in humans and laboratory model species, Lozano (1994) suggested that carotenoid-based ornaments may evolve as honest indicators of health because carotenoids *per se* are essential during the immune response. Carotenoids have been shown to enhance the immune system by different means, mostly because of their antioxidant function (Bendich 1989; Bendich 1993; Chew and Park 2004). The conjugated system of carotenoids allows them quenching reactive oxygen species (*i.e.,* free radicals like superoxide, hydroxyl, and nitric oxide, as well as non-radical oxidants like hydrogen peroxide, hypochlorous acid, and singlet oxygen; Rock et al. 1996). Unpaired electrons of reactive oxygen species make them very prone to oxidize biologically relevant molecules like DNA, proteins, and lipids, dam-

aging essential structures (*e.g.*, cell membranes) and functions (*e.g.*, T- and B-lymphocyte immune reactions; Halliwell 1994; Seifried et al. 2007). During the immune response, the phagocytic activity of macrophages and neutrophils overproduces reactive oxygen species (Chew and Park 2004). By scavenging these reactive oxygen species, carotenoids protect immune cells from potential damage and thereby enhance immune response. Individuals therefore face a trade-off between investing carotenoids into immune function or ornamentation. Thus, only healthier individuals may benefit from investing more carotenoids into ornamental coloration whereas individuals in poor health status may not afford such investment without incurring the costs for their self-maintenance. Cheating is prevented by the dual function of carotenoids although it is still necessary to invoke the assumption that carotenoids are a finite resource and, thus, that animals (or, at least, not all) cannot obtain enough carotenoids for both health and ornamentation.

The relationship between health and reactive oxygen species not only applies to the immune response. Several essential functions, like mitochondrial respiration and metabolic reactions, also produce free radicals and non-radical oxidants. When the imbalance between prooxidant and antioxidant molecules favour the former, organisms suffer oxidative stress (Costantini 2008). High levels of oxidative stress result in cumulative damage of essential molecules and functions that finally impair health and condition (Finkel and Holbrook 2000; Dowling and Simmons 2009). In addition to immune impairment, high levels of oxidative stress result in impaired reproduction (male fertility; Blount et al. 2001; Aziz et al. 2004, embryo viability; Bize et al. 2008, female and male reproductive effort; Alonso-Alvarez et al. 2006; Bertrand et al. 2006a; Costantini 2008, growth, performance; review in Metcalfe and Alonso-Alvarez 2010). Individuals are suggested to face a trade-off between allocating carotenoids for ornamentation and for the reinforcement of antioxidant capacity and, hence, for the maintenance of essential functions susceptible to oxidative stress (Pérez-Rodríguez 2009). Carotenoid-based ornaments may therefore indicate health and condition in a broader sense than merely immune capacity (von Schantz et al. 1999).

Studies supporting that carotenoids act as immune enhancers and antioxidants and that carotenoid-based ornaments signal immune and antioxidant capacity have accumulated in past few years (Blount et al. 2003; Faivre et al. 2003; McGraw and Ardia 2003; Saino et al. 2003; Alonso-Alvarez et al. 2004; Bertrand et al. 2006a; McGraw et al. 2006b; Clotfelter et al. 2007; Cucco et al. 2007; Alonso-Alvarez and Galván 2011; Peters et al. 2011), although quite a few studies also question the relevance of carotenoids as immune enhancers and especially as antioxidants (Saks et al. 2003b[1]; Costantini et al. 2006; Hõrak et al. 2006; McGraw et al. 2006c; Costantini et al. 2007; Dijkstra et al. 2007; Hõrak et al. 2007; Smith et al. 2007; Isaksson and Andersson 2008; Pérez-Rodríguez et al. 2008; Cohen and McGraw 2009; Pap et al. 2009; Vinkler et al. 2012). Recently, Costantini and Møller (2008) conducted a meta-analyses to specifically

[1]We considered Saks et al. 2003 to yield contradictory results on the relationship between carotenoid-based ornaments and immunity because their manipulation correlates with variables that are unlikely to be related with carotenoids deposited in the plumage. They reported that "[g]reenfinches with brighter yellow breast feathers showed stronger humoral immune response". However, the brightness parameter used in the study ("the sum of reflectances at all measured wavelengths between 550 and 625 nm") is predicted to negatively correlate with carotenoid feather content or, as shown in other study with greenfinches, to show no correlation (Saks et al. 2003a).

test the hypothesis that carotenoids function as antioxidants and concluded that carotenoids account for less than 0.002 % of total antioxidant capacity of birds. As a consequence of this study, several reviews have been published to "re-evaluate the antioxidant role" of carotenoids and its consequences for the evolution of carotenoid-based ornaments (Catoni et al. 2008; Monaghan et al. 2009; Pérez-Rodríguez 2009; Vinkler and Albrecht 2010). Most authors acknowledge that current evidences provide only weak support that carotenoids are essential antioxidants in species with carotenoid-based ornaments. However, it has been suggested that the observed conflicting findings may be the result of our still limited comprehension of the potential interactions between carotenoids and the other components of the antioxidant system and, hence, that it may be too soon to completely discard that carotenoids serve as *in vivo* antioxidants (Pérez-Rodríguez 2009).

Hartley and Kennedy (2004) formulated a different hypothesis (the 'protection' hypothesis) to explain how carotenoid-based ornaments are related to individual antioxidant capacity. They defended that carotenoids do not function as *in vivo* antioxidants but that they are sensible to reactive oxygen species (Palozza 1998), which oxidize them into toxic aldehydes that impair cell viability and destroy carotenoid pigment capacity. Hartley and Kennedy (2004) proposed that antioxidant compounds, like vitamin E or C, protect carotenoids from oxidation and preserve their pigment function. In this scenario, more intense colorations indicate that individuals efficiently protect carotenoids from oxidation and, hence, that they posses low levels of reactive oxygen species or a more efficient antioxidant system. Recently, this hypothesis has received some empirical support (Bertrand et al. 2006b; Pike et al. 2007; Pérez et al. 2008). In these studies, carotenoid-based ornaments positively responded to the administration of dietary antioxidants like vitamin E, vitamin C, and melatonin, which suggests that they protect carotenoids and make them available for ornamentation. However, these findings could be also interpreted as carotenoids being spared from their antioxidant role and substituted by the ingested antioxidants (Svensson and Wong 2011). Therefore, the fact that dietary antioxidants enhanced carotenoid-based ornaments does not necessarily indicate that carotenoids need protection from antioxidants.

Hormones and carotenoid-based signals.—Folstad and Karter (1992) proposed that androgens like testosterone could assure the honesty of the health-based signalling system proposed by Hamilton and Zuk (1982). They pointed out that the development, maintenance, and/or display of exaggerated ornamental traits depends on elevated testosterone levels, which in turn impair health by suppressing immune function and increasing oxidative stress levels (Peters 2007; Alonso-Alvarez et al. 2008). Testosterone may therefore lead to a physiological trade-off between ornamental investment and self maintenance that individuals resolved according to their health status (1992; Peters 2007). Thus, only high quality individuals are predicted to be able to withstand high testosterone levels to produce more exaggerated displays without compromising their own health.

Folstad and Karter (1992) therefore provided with a model for the evolution of carotenoid-based ornaments (at least, for those based on testosterone or other androgens) that

does not assume that carotenoids are health mediators or a limited resource (Pérez-Rodríguez et al. 2006). However, it has been suggested that the potential health benefit of carotenoids could play a key role in the evolution of health- and androgen-dependent ornaments by reinforcing signal honesty. Several studies show that high levels of testosterone enhance plasma carotenoid availability (Blas et al. 2006; McGraw et al. 2006a), which has been suggested to evolve as an adaptation to counteract deleterious effects of high testosterone levels (Blas et al. 2006; McGraw and Ardia 2007; Svensson and Wong 2011). Carotenoids may therefore constitute a physiological mechanism determining the extent to which animals can elevate testosterone levels and produce ornamental traits (McGraw and Ardia 2007). Thus, only animals with high carotenoid levels may be able to physiologically offset health costs resulting from high testosterone levels needed to become more colorful (Blas et al. 2006; McGraw and Ardia 2007; Svensson and Wong 2011).

Stressors and stress-related hormones have been suggested to create a physiological tradeoff between the use of carotenoids for ornamentation and self-maintenance (Bortolotti et al. 2009). As a consequence of stressful conditions, different hormones (*e.g.*, glucocorticoids like corticosterone) are secreted to induce physiological and behavioral adaptations that promote immediate survival (Nelson 2005; Cote et al. 2006). Glucocorticoids modulate immune capacity (Dhabhar and McEwen 1997; McEwen et al. 1997; McEwen 1998) and increase oxidative stress (Lin et al. 2004; Lin et al. 2006). Thus, glucocorticoids are suggested to increase the demand for carotenoids as antioxidants, limiting the amount of carotenoids available for ornamentation (Loiseau et al. 2008). Carotenoid-based ornaments may therefore signal the ability of individuals to cope with negative effects of glucocorticoids and, hence, individual capacity to recover from stressful environmental factors or to avoid potential stressful situations.

Vitamin A.—Some carotenoids mainly serve as metabolic substrate for the synthesis of retinol (vitamin A; Surai 2002). Provitamin A carotenoids include β-carotene and β-cryptoxanthin and most of their stereoisomers (Simpson 1983; Castenmiller and West 1998). Lutein, zeaxanthin, astaxanthin, and canthaxanthin are non-provitamin A carotenoids in mammals but not in other taxa (Simpson 1983; Castenmiller and West 1998). Fish and birds synthesize retinol from astaxanthin, canthaxanthin, and other oxocarotenoids (canary xanthophylls) and fish synthesize vitamin A in the form of dehydroretinol (vitamin A_2) from lutein (Bendich and Olson 1989; Olson 1989). Vitamin A results essential for health maintenance and normal functioning of different biological functions, mostly because of their ability to regulate gene expression and enzyme activity (Balmer and Blomhoff 2002; Blomhoff and Blomhoff 2006). Vitamin A acts as an immune enhancer by promoting differentiation and function of phagocytes and T- and B-lymphocytes (review in Kim 2011, Ross et al. 2011, and Mora et al. 2008), it controls embryonic growth and development by enhancing proliferation of different cell types (Edem 2009), and it constitutes the chromophore of the retinal visual pigments (Pepe 1999).

The relevance of carotenoid provitamin A activity has been rarely considered (Leclaire et al. 2011; Navarro et al. 2011), despite of its potential effects for the development and maintenance of carotenoid-based ornaments. First, nutritional requirements for vitamin A determine

carotenoid uptake affecting the amount of circulating carotenoids as well as their profile (von Lintig 2010). Carotenoids are metabolized to retinol in the enterocytes and, hence, just after absorption (Castenmiller and West 1998). Carotenoids are liberated to the blood according to individual needs for vitamin A, which therefore determines the amount and the species of carotenoids available for other functions. Second, vitamin A may also mediated immune enhancing effects of dietary carotenoids. This idea has been suggested by some authors which, at the light of evidences questioning the antioxidant capacity of carotenoids, defended that the immune enhancing effects of carotenoids could be the result of the immune enhancing effects of vitamin A (Hartley and Kennedy 2004; Cohen and McGraw 2009).

Costs Associated with Display

Carotenoid-based ornaments may entail different costs associated with display. First, conspicuousness of carotenoid-based ornaments may attract the attention of potential predators and, thus, individuals displaying more intense ornaments may suffer higher predation risk. Costs associated with predation have been extensively investigated in the Trinidadian guppy, *Poecilia reticulata*. Fish predators, like the cichlid *Aequidens pulcher*, preferentially attack more colourful guppies, even if duller guppies are closer (Godin and McDonough 2003). Consequently, Trinidadian guppies are selected for less conspicuous, better background-matching colorations in high-predation risk populations and for more conspicuous and more sexually attractive colorations in low-predation risk populations (Endler 1980). Within a single population and, hence, under the same predation regime, male Trinidadian guppies displaying more intense colorations also show a more efficient anti-predatory behavior, indicating that females may use male coloration to asses male viability (Godin and Dugatkin 1996). Predation-related costs in other species than Trinidadian guppies have been rarely investigated (for an example in birds see; Götmark and Olsson 1997) and the few available studies do not provide coincident evidences. For instance, in birds, it has been observed that carotenoids may be positively or negatively associated with flight performance (Blount and Matheson 2006; Huggins et al. 2010) and that no relationship may exist between carotenoids and anti-predatory behaviour (Cucco et al. 2006).

Because carotenoid-based ornaments of some species serve as signals of social status (review in Santos et al. 2011), it has been hypothesized that potential costs arising from antagonistic interactions may prevent cheating. In this context, interactions between individuals serve as subsequent tests of signal honesty where faking condition or fighting ability may incur large costs (Wong and Candolin 2005). Only males with superior condition or fighting ability can display elaborate ornaments without incurring extra costs. In different species, individuals with more intense carotenoid-based ornaments have been observed to be dominant over duller males (*e.g.*, Evans and Norris 1996; Senar and Camerino 1998; Pryke et al. 2001; Pryke et al. 2002; Pryke and Andersson 2003; Griggio et al. 2007). However, there is no clear demonstration of how cheating is controlled in systems where carotenoid-based ornaments signal social status. Candolin (Candolin 2000a; Candolin 2000b) showed that male three-spined sticklebacks, *Gasterosteus aculeatus*, with lower condition reduced their coloration right after interacting with males of higher

condition. Such colour changes improved signal honesty, measured as the strength of the correlation between male coloration and hatching success of male offspring. However, these studies cannot discern whether costs associated with male-male interactions or other different mechanisms (*e.g.*, trade-offs between immediate and future reproduction; Candolin 2000a) accounted for colour adjustment in low-quality males. Therefore, studies showing detrimental effects of deception as observed for other status signalling systems (Møller 1987; Nakagawa et al. 2008) are still needed.

Other potential costs that may arise from display have been suggested although they have rarely been investigated. For instance, conspicuousness of carotenoid-based ornaments may be also costly by decreasing hunting efficiency (Grether and Grey 1996), by attracting more parasites (Pérez et al. 2011), by revealing fluctuating asymmetry, by decreasing thermoregulatory capacity (Lindstedt et al. 2009), or, in birds, by decreasing plumage resistance (Delhey et al. 2011).

Alternatives to Condition-dependent Signalling

Carotenoid-based ornaments may evolve in contexts different than condition-dependent signalling, although these alternative contexts have usually been less investigated. Nevertheless, the study of carotenoid-based ornaments in different contexts may provide new insights into our general understanding of carotenoid-based ornaments as well as a deeper understanding of why and how carotenoid-based ornaments evolved as condition-dependent signals. Below, we introduce some alternative functions of carotenoid-based ornaments.

In some species, carotenoid-based colours serve as UV filters that protect individuals from deleterious effects of UV radiation (Hairston 1976; Rothschild et al. 1986; Proctor and Garga 2002; Moeller et al. 2005; Rautio et al. 2009). For instance, copepods adjust their coloration according to UV intensity, increasing their pigmentation with increasing UV intensity (Byron 1982; Hansson 2004; Garcia et al. 2008). Carotenoid-based colorations also serve as aposematic signals in invertebrates (Britton et al. 1977; Proctor and Garga 2002; Bezzerides et al. 2007) and may play a similar role in some reptiles, amphibians, and birds (review in Blount and McGraw 2008). Recently, it has been shown that the expression of carotenoid-based colorations functioning as aposematic signals may also depend on individual condition (Blount et al. 2012).

Discrete colour variation may result from differential carotenoid deposition, which clearly contrasts with continuous variation associated with condition-dependent signalling (see Fig. 2 in (Dale et al. 2001). To our knowledge, only one study, conducted in red-billed queleas, *Quelea quelea*, has shown that carotenoids may account for discrete colour variation (Dale 2000). In this species, colour has been suggested to function as a signal of individual identity in social contexts where recognition is crucial (*e.g.*, dominance hierarchies, neighbour-stranger recognition, kin recognition, or delayed reciprocal altruism; Dale et al. 2001). As observed for other types of coloration, for instance those based on melanins or pteridines, discrete colour phenotypes may also function in other contexts like in social status signalling (Greenberg and Crews 1990), as signals driving assortative mating (Nosil 2007; Pryke and Griffith 2009), or as signals of alternative life-

history strategies (Gross 1996; Blount and McGraw 2008; Olsson et al. 2008).

Colour polymorphisms associated with alternative life-history strategies appear to be wide spread in insects (Svensson et al. 2005), birds (Tuttle 2003), fish (Dijkstra et al. 2009), and reptiles (Sinervo and Lively 1996; Huyghe et al. 2010). Colour polymorphisms usually correlate with suites of different phenotypic traits and, thus, distinct colour morphs show distinct morphological (Huyghe et al. 2009a), physiological (Sinervo et al. 2000a; Huyghe et al. 2009b), and behavioural traits (Sinervo and Lively 1996). Each colour morph may further reflect different reproductive strategies among individuals of the same sex, like territorial, altruistic, and sneaky male behaviours (Sinervo and Lively 1996) or *r*- and *k*- reproductive strategies in females (Sinervo et al. 2000b). Maintenance of colour polymorphism is suggested to result from different evolutionary processes that prevent colour allele fixation and, thus, that avoid dominance of a single strategy (review in Gray and McKinnon 2007 and McKinnon and Pierotti 2010). Colour polymorphisms are thus suggested to result from disruptive selection (*i.e.*, selection for extreme morphs over intermediate forms), negative frequency-dependent selection (*i.e.*, selective advantage of rare morphs), or from migration for divergent environments. Genetic drift, despite of its depleting effects on genetic variation and of promoting random fixation of alleles, has also been suggested to play an important role in the maintenance of colour polymorphism, always in combination with some of the selective forces named above (Runemark et al. 2012).

Discrete colour morphs are suggested to show lower phenotypic plasticity and tighter genetic determination (Sinervo et al. 2001; Sinervo et al. 2006). Colour polymorphisms are suggested to have a relatively simple genetic basis given that they commonly segregate as a single Mendelian locus (although see Calsbeek et al. 2009 for a more complex genetic determination). Although the genetic basis of colourful traits is likely to be under the control of several loci, these may behave like a single locus because of the high genetic correlations that may exist among them owing to linkage disequilibrium (or gametic-phase disequilibrium; Sinervo and Calsbeek 2006). The relative contribution of environmental and genetic components to colour expression constitutes the main difference between polymorphic and condition-dependent colour traits. Thus, in contrast to discrete colour morphs, condition-dependence traits are suggested to show a higher phenotypic plasticity, given that they covary with condition, which substantially depends on genotype-environment interactions (Hill 2011).

In some cases, carotenoid-based coloration may be non-functional. For instance, carotenoid-based coloration in females or younger and non-reproductive individuals are suggested to be the by-product of selection on adult males and, hence, to result because of genetic correlation. For instance, adult male coloration in great tits (*Parus major*) may be important for sexual selection (Helfenstein et al. 2010) and juvenile coloration may simply exist because no selective pressure may hinder the expression of carotenoid-based ornaments at this age (*i.e.*, not costly to produce for juveniles because carotenoids are provided by parents (Fitze et al. 2003a), no predation costs in the nest, and no advantages/disadvantages after fledging (Fitze and Tschirren 2006).

Carotenoids and Lizard Coloration

Condition-dependent signalling through carotenoid-based ornaments has been suggested to be taxonomically widespread given that carotenoids occur in most ecosystems and the likely taxonomical conservation of the dietary, hormonal, and health mechanisms suggested to mediate honesty of these traits (Andersson 1986; Peters 2007; Sullivan and Kwiatkowski 2007). However, to date, most studies are based on birds and fish whereas other vertebrate groups have been almost neglected. In lizards, only a handful of studies have been conducted in the past few years (Kwiatkowski and Sullivan 2002; Steffen and McGraw 2007; Olsson et al. 2008; Steffen and McGraw 2009; Weiss et al. 2011). Male and female lizards present a wide diversity of colourful ornaments, which are used in different sexual and social contexts (Stuart-Fox and Ord 2004; Nicholson et al. 2007; Stuart-Fox and Moussalli 2008). Carotenoids are commonly found in the lizard integument (Fox 1947; Cooper and Greenberg 1992) although the extent to which carotenoids determine coloration in lizards is only well known in a few species, most of them anoline lizards (Ortiz et al. 1963; Macedonia et al. 2000; Olsson et al. 2008; Steffen and McGraw 2009).

As in fish and amphibians, colour production in lizards relies on the dermal chromatophore unit (Bagnara et al. 1968). The dermal chromatophore unit is a distinct morphological and physiological structure of the integument that comprises the different integumentary cell types that interact with light, *i.e.* the chromatophores (Fig. 4; Bagnara et al. 1968; Taylor and Hadley 1970, review in Cooper and Greenberg 1992). In lizards, three types of chromatophores form the dermal chromatophore unit. Right beneath the epidermis and the basal lamella, there exists a layer of xanthophores and erythrophores containing yellow-orange carotenoid and pteridine pigments as well as colourless pteridines (Morrison et al. 1995). Xanthophores and erythrophores contain carotenoids, pteridines, or both, and they are merely distinguished by colour, the latter being redder than the former. Carotenoids are usually present in small droplets whereas pteridines are included in larger specific organelles, pterinosomes. Below the xanthophore and erythrophore layer, lizards present a layer of iridophores, which contain sacks of crystal platelets

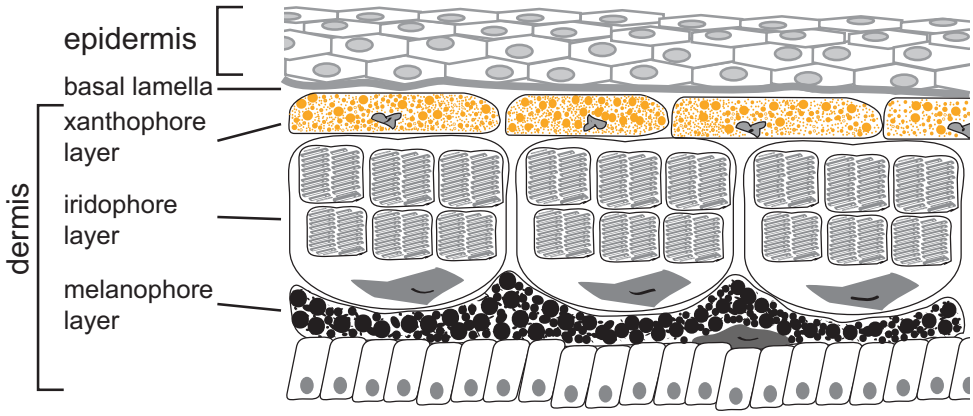

Fig. 4.—The dermal chromatophore unit sensu Bagnara et al. (1968).

made up of purine, mainly guanine. Beneath the iridophore layer, there is a layer of melanophores, which contain melanin pigments in specific organelles named melanosomes.

Lizard coloration results from the combined absorbing and reflecting effects of the distinct chromatophores (Grether et al. 2004). Incident light first encounters the xanthophore and erythrophore layer. As light passes through this layer, carotenoid and pteridine pigments subtract (absorb) short-wavelength light. Thus, only a fraction of light successfully reaches the iridophore layer. Iridophores produce different reflective effects depending on the arrangement of their purine crystal platelets (Menter et al. 1979). When crystals are arranged in subsequent parallel layers, light is reflected by a phenomenon of multilayer interference that selectively reflect light in predominant wavelength range (Box 2; review in Huxley 1968; Land 1972; Kinoshita and Yoshioka 2005; Parker 2009) and produce iridescence (*i.e.,* colour changes with viewing angle; (Doucet and Meadows 2009). When crystals are randomly distributed, light scattering mechanisms govern light reflection in the iridophore layer (Menter et al. 1979). In such circumstances, iridophores may reflect light of all wavelengths (*i.e.*, white light) or selectively reflect blue-green light and transmit long-wavelength light (*i.e.*, Tyndall blue). Finally, light that it is not reflected by iridophore platelets is transmitted to the melanophore layer, where melanins absorb it. Lizard melanophores only contain eumelanin, which absorb light of all wavelengths, and, in contrasts with mammals and birds, lizards do not synthesize red phaeomelanins (Bagnara and Hadley 1973).

Lizard coloration reflects different aspects of animal quality that may be related with carotenoids. In some lizard species, coloration correlates with immune capacity (Olsson et al. 2005; Martín et al. 2008; López et al. 2009; Martín and López 2009; Ruiz et al. 2011), parasite load

Box 2. Multilayer interference in iridophores

Iridophores can selectively reflect light and, hence, produce colour when purine crystals of iridophores are regularly arranged in parallel layers, given that this particular arrangement fulfils the conditions for multilayer interference. Light passing through iridophore platelets alternately encounters layers of high (purine crystals) and low (cytoplasm) refractive indexes, whose thicknesses are small enough (~200 nm) to be comparable with the range of light wavelengths (400-700 nm). Each wavelength of light is reflected at every crystal-cytoplasm interface and its phase changes as it passes from low to high refractive layers (Fig. I). Phase change may result in constructive interference or destructive interference of the reflected wavelengths (Fig. I). Constructive interference results in the superposition of waves with same wavelength and thus in a wave of higher amplitude (higher intensity) whereas destructive interference results in a wave of lower amplitude (lower intensity). Thus, iridophores reflect light with substantial modifications in the contribution of each wavelength, which gives the appearance of colour. Crystal thickness and spacing as well as refractive indexes of cytoplasm and purine crystals determine which wavelengths are constructively or destructively interfere by iridophores.

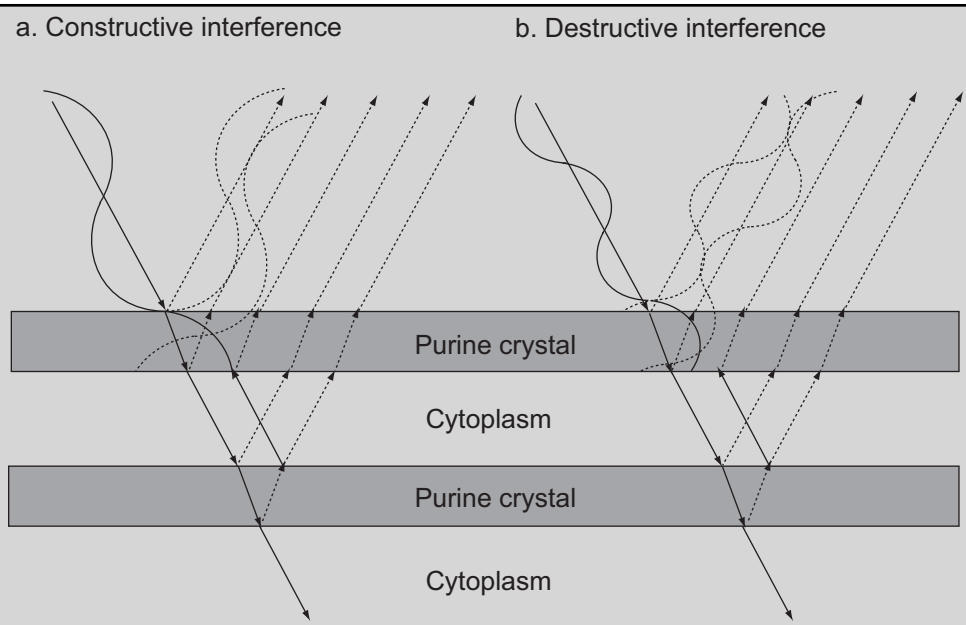

Fig. I.—Constructive (**a**) and destructive (**b**) interference in iridophore platelets. Continuous lines represent incident light and discontinuous lines represent reflected light.

(Stapley and Keogh 2006; Weiss et al. 2006; Calisi et al. 2008), antioxidant levels (Martín and López 2010), oxidative damage (Olsson et al. 2008; Cote et al. 2010; Weiss et al. 2011), and testosterone and corticosterone levels (Salvador et al. 1996; Calisi and Hews 2007). However, the presence of different integumentary chromatophores as well as the absence of studies specifically testing the origin of colour variation makes confirmation that carotenoids are actually mediating the link between coloration and quality in lizards difficult. Moreover, there is no evidence that coloration in lizards depends on carotenoid intake. Studies conducted in four different and taxonomically distant lizard species found no effect of dietary carotenoid supplementation (Olsson et al. 2008; Steffen et al. 2010; Weiss et al. 2011) and nutritional stress (Cote et al. 2010; Steffen et al. 2010) on coloration. These studies therefore question that lizard coloration is limited by carotenoid availability, suggesting that different mechanisms than those reported in bird and fish may assure honesty of condition-dependent signalling or that carotenoid-based ornaments may serve for different purposes in lizards.

Lacerta vivipara : A Model Species for the Study of Carotenoid-based Signals

Some Notes on the Phylogeography and Ecology of *Lacerta vivipara*

The common lizard, *Lacerta vivipara*[2] Jacquin, 1787 is a small ground-dwelling lizard of the family Lacertidae (Sauropsida: Squamata). It is the reptile with the widest geographic distribution of the world. It lives all across Eurasia, from Ireland and the Northwestern Iberian Peninsula in the west, to the Hokkaido and Sakhalin islands (Japan and Russia, respectively) in the east, and from Scandinavia in the north, to Northern Spain and Macedonia in the south (Fig. 5A). Within its distributional range, up to six distinct clades have been described according to differentiation in mitochondrial DNA, karyotype, and reproductive mode (table 2; Surget-Groba et al. 2001; Surget-Groba et al. 2002; Surget-Groba et al. 2006). Several subspecies have been described from these clades (table 2). However, none of them seems to be widely accepted because they have been observed to hybridize in both laboratory and natural conditions (Heulin et al. 1992; Lindtke et al. 2010). Populations used in the present study were located in the Western and Central Pyrenees and in the Cévennes (Massif Central, France). They respectively belong to the western oviparous clade and to the western viviparous clade (Fig. 5B; Surget-Groba et al. 2001). Some external differentiation, mainly in scalation and female coloration, has been observed between the Western oviparous clade and the western viviparous clade although no external trait clearly differentiates among them (Arribas 2009).

Lacerta vivipara commonly inhabits peat bogs and humid heathlands (Fig. 5C-D; (Strijbosch 1988; Hermida Lorenzo and Lamas Anton 2005; Covaciu-Marcov et al. 2008; Farren et al. 2010). Humidity seems to be its most limiting environmental factor (Ceirans 2007) because it importantly affects growth and survival of lizards (Grenot et al. 1987; Sorci et al. 1996; Lorenzon et al. 1999; Lorenzon et al. 2001; Marquis et al. 2008; Le Galliard et al. 2010). *Lacerta vivipara* is highly susceptible to changes in humidity because of its highly permeable skin (Grenot et al. 1987). Humidity has therefore been suggested to determine the distribution of *L. vivipara*, especially in southern populations. On the Iberian Peninsula and in Southern France, its geographic distribution is restricted to mountainous habitats and lowlands of Atlantic influence and it is absent in zones of Mediterranean influence where more xeric conditions prevail (Perez-Mellado 1998).

[2]**Nomenclatural note:** Recent taxonomic reviews split *Lacerta* into 16 different genera (Arnold 2007), assigning the common lizard to the monospecific genus *Zootoca* Wagler, 1830 (Mayer 1996). However, the phylogenetic position of the common lizard remains unresolved given that still no robust systematic of the Lacertidae exists (Harris et al. 1998; Pavlicev and Mayer 2009). Available studies infer distinct topologies within Lacertidae and suggest different groups (none of them supported) as the closest relative to the common lizard; *e.g. Lacerta sensu stricto* (*i.e.*, *L. agilis* group; Mayer 1996), *Podarcis* (Pavlicev and Mayer 2009), *Archeolacerta* (Fu 2000; Kapli et al. 2011), *Phoenicolacerta*, or *Takydromus* (Arnold 2007). Therefore, although we acknowledge that the genus *Lacerta* (*s.l.*) may be polyphyletic and that the common lizard might belong to a different genus, we prefer to use in the present study the more conservative name of *Lacerta vivipara* instead of *Zootoca vivipara*.

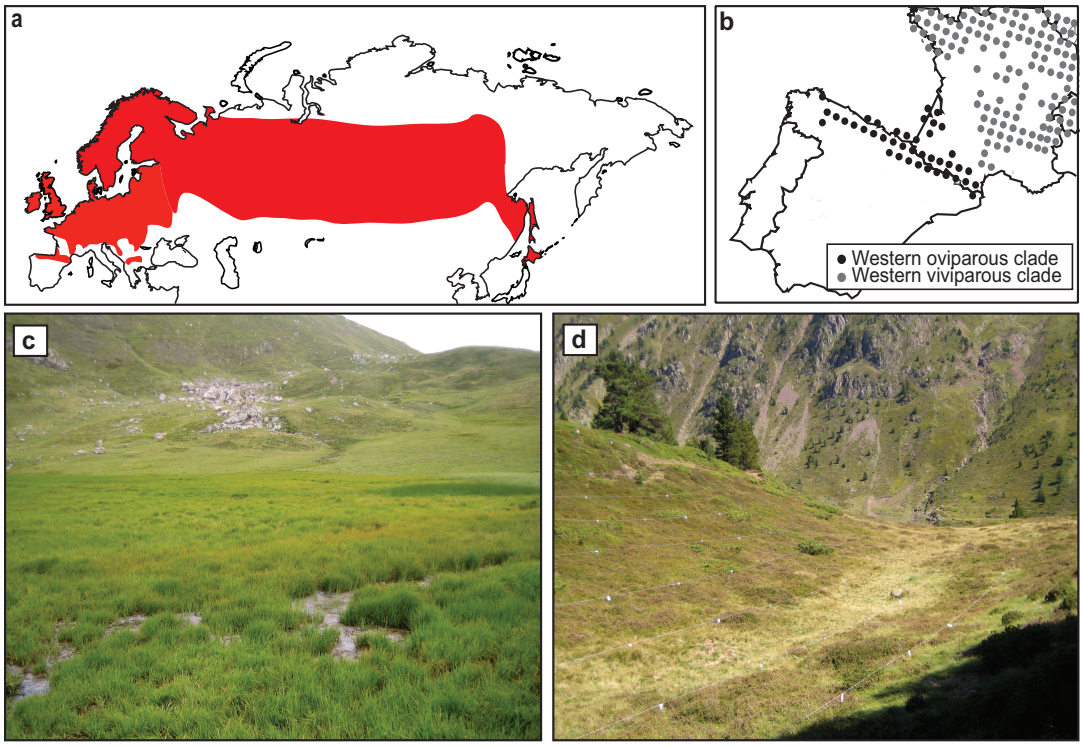

FIG. 5.—Distribution and habitat of *Lacerta vivipara*. **a**. World distribution of *L. vivipara* (Surget-Groba et al. 2006). **b.** Iberian and French distribution of the Western oviparous clade (50 x 50 km, UTM map; Gasc et al. 1997). **c.** Typical peatbog habitat of *L. vivipara* in the Pyrenees (Portalet, Huesca, Spain). **d.** Typical heathland habitat of *L. vivipara* in the Pyrenees (Somport, Huesca, Spain).

Lacerta vivipara preys on small invertebrates and its prey spectrum resembles environmental availability (Heulin 1986). It mainly preys on homoptera, araneae, and diptera, which together represent 63.25 % ± 3.78 of the total preys consumed (mean from N = 14 populations; Avery 1962; Koponen and Hietakangas 1972; Pilorge 1982; Heulin 1986; Khodadoost et al. 1987; Perez-Mellado 1998; Roig et al. 1999; Kuranova et al. 2005; Zhao et al. 2006). The remaining percentage is formed by an average of 9.1 ± 0.8 different animal groups, including insects (25.4 % ± 2.9 of the total preys consumed) and non-insect taxa (oligochaeta, isopoda, gastropoda, myriapoda, collembola, acari, and opiliones; 6.5 % ± 1.4 of the total preys consumed).

Lizards enter hibernation between September and November (Perez-Mellado 1998) and emerge between February and April depending on latitude and altitude (Heulin et al. 1994; Roig et al. 2000). During hibernation, lizards withstand subzero temperatures, surviving even if they partially freeze (Voituron et al. 2004). Males emerge two to four weeks before females, to allow sperm maturation (Courty and Dufaure 1979; Courty and Dufaure 1980; Roig et al. 2000). Mating occurs immediately after females emerge from hibernation (Bauwens and Verheyen 1985). During the mating season, antagonistic interactions between males occur in order to control access to females (Heulin 1988; Gonzalez-Jimena and Fitze 2012). Female mate choice and, hence, intersexual selection mechanisms additionally determine mating success in *L. vivipara*

Table 2—Main mitochondrial, reproductive, and karyological differences between clades of *Lacerta vivipara*. Clades described for *Lacerta vivipara* populations based on differences in mitochondrial DNA, reproductive mode, in the number of chromosomes of male and females, and in the position of the centromere in the female W chromosome. Geographic distribution of each clade and described subspecies are also given (for more details on distribution see Fig. 1 in Surget-Groba et al. 2006).

Clade	Mitochondrial clade*	Reproductive mode	Male/female chromosomes	W chromosome	Distribution	Subspecies name
Eastern oviparous	Clade A	Oviparous	36/36	acrocentric	Slovenia, Croatia, Southern Austria, Northern Italy	*L. v. carniolica*
Western oviparous	Clade B	Oviparous	36/35	acrocentric	Northern Spain, Southern France	*L. v. louislantzi*
Central viviparous I	Clade C	Viviparous	36/35	acrocentric	Northwestern Hungary and Eastern Austria	*L. v. vivipara*
Central viviparous II	Clade F	Viviparous	36/36	acrocentric	Central Hungary and Central Austria	—
Eastern viviparous	Clade D	Viviparous	36/35	acrocentric	Asia and Eastern Europe	*L. v. sachalinensis*
Western viviparous	Clade E	Viviparous	36/35	submetacentric	Northwestern Europe and Carpathians	—

**sensu* Surget-Groba et al. (2006)

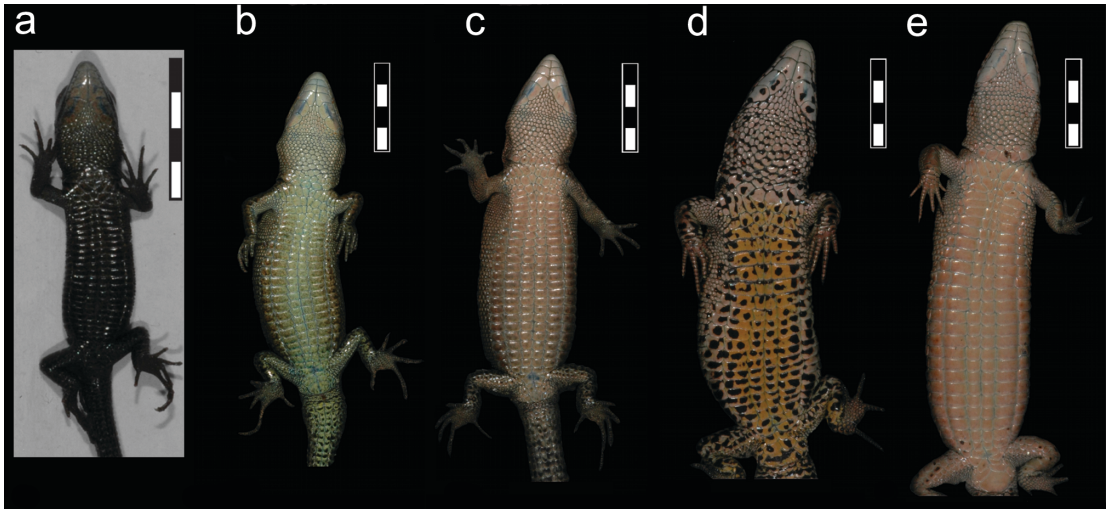

Fig. 6.—Ontogenetic changes in the ventral coloration of *L. vivipara*. Ventral coloration of a juvenile (**a**), yearling male (**b**), yearling female (**c**), adult male (**d**), and adult female (**e**).

(Fitze et al. 2008). *Lacerta vivipara* is a polygynandrous species (Fitze et al. 2010). Females lay clutches sired by an average of two different males and males sire clutches of an average of four different females (Fitze et al. 2005). Females lay 3 to 13 eggs after three to six weeks of gestation (Avery 1975; Pilorge 1987; Perez-Mellado 1998). In viviparous populations, females lay eggs with a translucent shell and juveniles hatch within two hours after egg laying (Heulin et al. 1992). In oviparous populations, female lay eggs with a calcified shell and juveniles hatch after an incubation period of 30 to 50 days (Heulin et al. 1992; Heulin et al. 1994). Females may produce up to three clutches (one at high altitude). In low altitude populations, juveniles become adult and reproductively active after the first hibernation and after the second or third hibernation in high altitude populations (Heulin et al. 1994; Perez-Mellado 1998). Female common lizards have been observed to live for up to 13 years whereas males live for up 4 years (Massot et al. 2011).

Ventral Coloration of *Lacerta vivipara*: Physiological and Behavioural Aspects

Juveniles of *Lacerta vivipara* are completely black at hatching and start developing adult coloration during their first year of life (Fig. 6). Male ventral coloration ranges from yellow to orange and spreads from the tail to the throat (Bauwens et al. 1987; Fitze et al. 2009). In the Western oviparous clade, male coloration ranges from white to yellow and orange and only in rare occasions it is present on the gular scales (Sinervo et al. 2007). Female ventral coloration ranges from cream to orange although in the Western oviparous clade female ventral coloration is never yellow or orange, and ranges from white to pink (Vercken et al. 2007; Cote et al. 2008; Arribas 2009). Arribas (2009) suggested that such colour differences might result because no yellow-orange pigments are present in the ventral coloration of females of the Western oviparous

clade. Male ventral coloration additionally shows an ultraviolet peak that is absent in females and it is commonly spattered with small dark spots that are absent (or exist in low numbers) in females (Bauwens et al. 1987). In *L. vivipara*, the dermal chromatophore unit shows the typical arrangement of chromatophore cells observed in most reptiles, fish, and amphibians (Breathnach and Poyntz 1966; Bryant et al. 1967). However, the relevance of these other chromatophores for colour determination is currently completely unknown. The ventral yellow-orange coloration of *Lacerta vivipara* may result from carotenoid deposition in the skin (Czeczuga 1980). Czeczguga (1980) analyzed the content of carotenoids in the skin, muscles, liver, and intestine of *L. vivipara* and confirmed the presence of different carotenoid species as β-carotene, β-cryptoxanthin, canthaxanthin, lutein, zeaxanthin, astaxanthin and diatoxanthin. However, whether the skin content of carotenoids determines the intensity of male ventral coloration is unknown. Similarly, whether pteridines also contribute to yellow-orange coloration of *L. vivipara* has not been tested yet.

The function of the ventral coloration of *L. vivipara* remains poorly understood. Early experimental studies suggested that ventral coloration may play an important role in sex recognition because males approached female-painted males as if they were females (Bauwens et al. 1987). More recent evidences suggest that ventral coloration could have additional functions. However, the appearance of these evidences has resulted in an intense debate about the correctness of two conflicting hypothesis (Cote et al. 2008; Vercken et al. 2008). On one hand, it has been suggested that ventral coloration *L. vivipara* is a continuous trait that is environmentally determined and that may function as a condition-dependent signal (Cote et al. 2008). Experimental manipulations demonstrated that ventral coloration was affected by several population parameters that affect a lizard's body condition, *e.g.,* population density (Meylan et al. 2007) and adult sex ratio (Cote et al. 2008). However, the ultimate mechanism of the observed colour changes, *i.e.*, whether differential skin carotenoid content or changes in other integumentary components account for such colour changes, has never been investigated.

On the other hand other studies suggested that ventral coloration in *L. vivipara* may be a discrete polymorphic trait that serves as a signal of alternative life-history strategies (Sinervo et al. 2007; Vercken et al. 2007). On one hand, Vercken et al. (2007, see also Vercken and Clobert 2008; Vercken et al. 2010) suggested the existence of three discrete colour morphs (orange, yellow, and mixed yellow-orange) in females of the western viviparous clade. They suggested that colour morphs may correlate with distinct reproductive and behavioural strategies, suggesting that morphs may affect social interactions and, more likely, antagonistic female-female interactions (Vercken and Clobert 2008). Sinervo et al. (2007) proposed that male (but not females; Arribas 2009) of the western oviparous clade show discrete colour morphs (see Fig. 7 for further details). As described for *Uta stansburiana* (Sinervo and Lively 1996), these male colour phenotypes could reflect alternative life-history strategies that are maintained through negative frequency dependent selection. These studies suggest that ventral coloration in *L. vivipara* is a polymorphic trait that may be under tight genetic control and that reflects differences in genetic background.

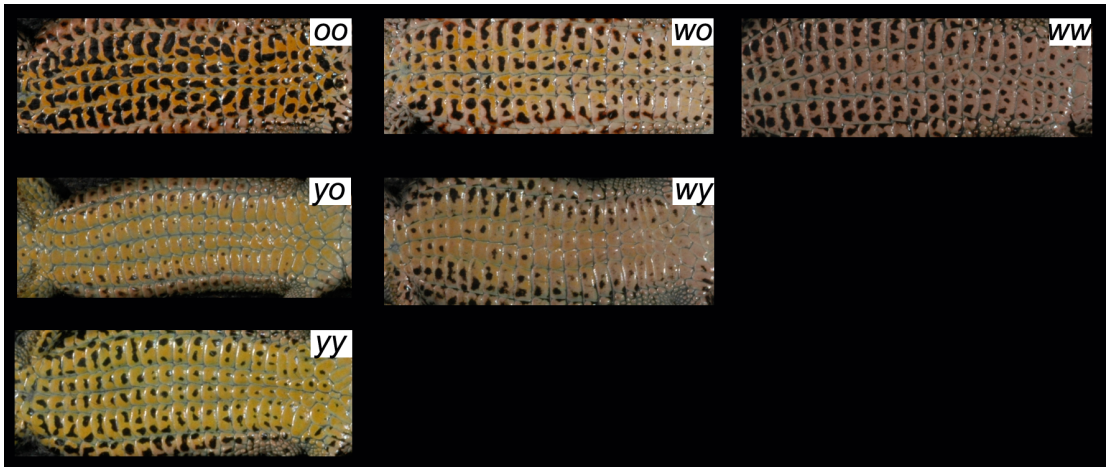

FIG. 7.—Morphotypes described in male common lizards of western oviparous populations (Sinervo et al.2007). Colour polymorphism has been suggested to be under control of a putative single trialelic locus. Three colour aleles; white (*w*), yellow (*y*), and orange (*o*) are combined into 6 distinct phenotypes, three putative homozygotes; orange-orange (*oo*), white-white (*ww*), and yellow-yellow (*yy*), and three putative heterozygotes; white-orange (*wo*), white-yellow (*wy*), and yellow-orange (*yo*).

Objectives

The general objective of this thesis is to provide new insights into the evolution of carotenoid-based signals. In contrast to birds and fish, where the evolutionary, behavioural, and physiological mechanisms associated with carotenoid-based signals are relatively well understood, lizards have been almost neglected. Consequently, there exists limited evidence for the generality of previous findings and proposed evolutionary mechanisms under which carotenoid-based signalling systems evolved. This thesis aims at filling this gap, by investigating the proximate mechanisms of lizard coloration using the common lizard, *Lacerta vivipara*, as a model system.

Previous studies on the ventral yellow-orange coloration of *Lacerta vivipara* suggest that carotenoids could indeed account for honest condition-dependent signalling in this species, which would provide support that the proximate mechanisms of carotenoid-based signalling are common in birds, fish, and lizards. In contrast, other studies suggest that carotenoids may account for colour polymorphism and, hence, show small environmental plasticity, which is in clear contrast to the hypotheses derived from studies in birds and fish. In order to disentangle between these two conflicting hypotheses, we particularly investigate the following questions:

Is it the ventral yellow-orange coloration of *L. vivipara* made up of carotenoids?—We investigate **i.** the profile and concentration of carotenoids in the skin of *L. vivipara* (**chapter II**, **III**), **ii.** the concentration of skin carotenoids in relation to the concentration of carotenoids in lizard reserve tissues (**chapter III**) **iii.** the relationship between skin content of carotenoids and measures of the ventral colouration (**chapter II, V**), and **iv.** the presence of other yellow-orange pigments (pteridines) in the ventral skin of *L. vivipara* (**chapter II**).

Is the ventral yellow-orange coloration of *L. vivipara* environmentally plastic?—We investigate whether dietary supplementation with carotenoids enhance the expression of the ventral coloration of *L. vivipara* of both, western viviparous populations (**chapter II**) and western oviparous populations (**chapter IV, chapter V**). We further investigate the potential interaction between dietary intake of carotenoids and the intake of naturally occurring dietary lipids (**chapter IV**). We also investigate whether the yellow-orange coloration of *L. vivipara* varies according to physiological stress (**chapter II**).

Are carotenoids or other integumentary components responsible for environmentally induced colour changes observed in *L. vivipara*?—We investigate whether carotenoids or other integumentary components (iridophores and melanophores) are responsible for the observed colour changes, and thus whether carotenoids are mediating the relationship between coloration and environmental conditions in *L. vivipara*, as suggested so far (**chapter V**).

Does the ventral-orange coloration of *L. vivipara* reflect alternative reproductive strategies? —We investigate whether **i.** skin carotenoid content is responsible for the described discrete male colour morphs (**chapter V**), and **ii.** whether male colour morph signals alternative mating strategies that are sustained by negative frequency dependent selection (**chapter VI**).

The mentioned chapters (except chapter VI) correspond to original manuscript published, accepted for publication, or under review in different international scientific journals. A general discussion of all chapters as well as a summary of the main conclusions is given in **chapter VII**.

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Chapter II

Carotenoid-based Colours Reflect the Stress Response in the Common Lizard

Fitze, P.S., Cote, J., San-Jose, L.M.,Meylan, S., Isaksson, C., Andersson, S., Rossi, J.-M., and J. Clobert. 2009. PLoSone 4 (4): e5111

Abstract: Under chronic stress, carotenoid-based colouration has often been shown to fade. However, the ecological and physiological mechanisms that govern colouration still remain largely unknown. Colour changes may be directly induced by the stressor (for example through reduced carotenoid intake) or due to the activation of the physiological stress response (PSR, *e.g.*, due to increased blood corticosterone concentrations). Here, we tested whether blood corticosterone concentration affected carotenoid-based colouration, and whether a trade-off between colouration and PSR existed. Using the common lizard (*Lacerta vivipara*), we correlatively and experimentally showed that elevated blood corticosterone levels are associated with increased redness of the lizard's belly. In this study, the effects of corticosterone did not depend on carotenoid ingestion, indicating the absence of a trade-off between colouration and PSR for carotenoids. While carotenoid ingestion increased blood carotenoid concentration, colouration was not modified. This suggests that carotenoid-based colouration of common lizards is not severely limited by dietary carotenoid intake. Together with earlier studies, these findings suggest that the common lizard's carotenoid-based colouration may be a composite trait, consisting of fixed (*e.g.*, genetic) and environmentally elements, the latter reflecting the lizard's PSR.

Key words: Blood carotenoid and corticosterone concentration; Carotenoid-availability; Emergency life history state; Honest signalling; Composite trait.

Los Colores Basados en Carotenoides Reflejan la Respuesta al Estrés en la Lagartija de Turbera

Resumen: A menudo, se ha observado que las coloraciones basadas en carotenoides palidecen bajo condiciones de estrés crónico. Sin embargo, los mecanismos ecológicos y fisiológicos que gobiernan la coloración permanecen profundamente desconocidos. Los cambios de color pueden ser inducidos directamente por el factor estresante (por ejemplo, a través de reducir la ingesta de carotenoides) o debidos a la activación de la repuesta fisiológica al estrés (RFE, *e.g.*, al aumentar los niveles de concentración de corticosterona). Aquí, testamos si la concentración sanguínea de corticosterona afecta a las coloraciones basadas en carotenoides y si existe un conflicto entre la coloración y la RFE. Utilizando la lagartija de turbera (*Lacerta vivipara*), mostramos de forma correlativa y experimental que los niveles elevados de corticosterona están asociados con coloraciones más rojas en el vientre de las lagartijas. En este estudio, los efectos de la corticosterona no dependieron de la ingestión de carotenoides, indicando la ausencia de un conflicto entre la coloración y la RFS por los carotenoides. Mientras que la ingesta de carotenoides aumentó la concentración sanguínea de carotenoides, la coloración no se alteró. Esto sugiere que la coloración basada en carotenoides de las lagartijas de turbera no está severamente limitada por la ingesta de carotenoides con la dieta. Junto con anteriores estudios, estos resultados sugieren que la coloración basada en carotenoides de la lagartija de turbera puede ser un carácter compuesto, formado por elementos fijos (*e.g.*, genéticos) y ambientales, estos últimos responsables de reflejar la RFS de las lagartijas.

Introduction

Colour signals are usually genetically and/or environmentally determined (Kodric-Brown 1989; Fitze et al. 2003; Tschirren et al. 2003; Hill and McGraw 2006; Fitze et al. 2007). However, while genetically determined elements signal an individual's life history strategy (see Sinervo et al. 2001 and Sinervo et al. 2007 for reptiles and Caesar et al. 2007 for insects), environmental variance stems from nutritional conditions (Kodric-Brown 1989; Fitze et al. 2003; Tschirren et al. 2003; Fitze et al. 2007). and health status (Milinski and Bakker 1990; Brawner et al. 2000; McGraw and Hill 2000). Environmental determination of many colour signals stems from differences in carotenoid deposition. Carotenoids are widely used colour pigments that cannot be synthesized by animals (Goodwin 1984) and thus, they must be obtained through feeding. Carotenoids are relevant to the immune system (Bendich 1989; Fitze et al. 2007) and they are thought to fulfil antioxidant functions (Blount et al. 2001; Alonso-Alvarez et al. 2004, but see Isaksson and Andersson 2008). These multiple functions may create trade-offs in carotenoid-limited animals. For example, during an immune challenge animals may favour using carotenoids for immune function rather than for colouration (Blount et al. 2003; McGraw and Ardia 2003; Fitze et al. 2007). Similarly, stress factors may divert the use of carotenoids from colouration by means of allostasis (Landys et al. 2006), possibly explaining why stressed animals sometimes exhibit reduced colouration (Fig. 1(ii); Milinski and Baker 1990; Belthoff et al. 1994; Brawner et al. 2000; Blount et al. 2001; Meylan et al. 2007; Cote et al. 2008). While the trade-off between carotenoid contribution to the immune system and colouration has frequently been investigated, the trade-off between stress and colouration has received much less attention. Indeed, the extent to which such trade-offs occur might depend on the role that colouration plays in a given species: signalling individual quality or individual strategy.

In response to a stress factor, vertebrates usually initiate a physiological stress response (PSR) by secreting glucocorticoids (Bentley 1998; Nelson 2005). During a PSR, many vertebrates release corticosterone from the adrenal glands (Axelrod and Reisine 1984), including birds, amphibians, reptiles and some mammals (Bentley 1998; Nelson 2005), and typically corticosterone concentrations rise within a few minutes in response to acute stress (for example social and agonistic interactions; Creel 2001). Corticosterone modifies vertebrate behaviour and physiology (increasing oxygen intake, decreasing pain perception, and enhancing sensory function and memory; Wingfield and Ramenofsky 1999; Moore and Jessop 2003; Nelson 2005). It also acts on the metabolic pathways to replenish the energy reserves used during stress (Nelson 2005; Cote et al. 2006), for example when escaping a predator. In addition, it increases locomotor performance (Cash and Holberton 1999; Cote et al. 2006), which may facilitate the possibility of finding new food sources (Cash and Holberton 1999), suggesting that at least during acute PSR corticosterone adaptively influences vertebrate behaviour (Wingfield and Sapolsky 2003). During acute stress, increased blood corticosterone levels are typi-

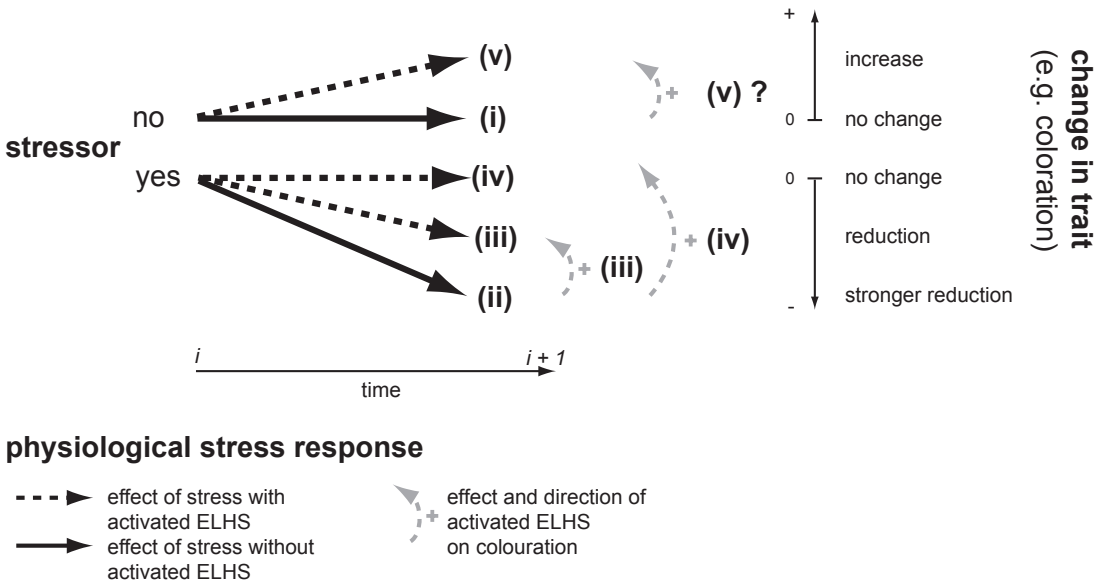

FIG. 1.—Effects of stress factors and PSR on colouration. The theory predicts that colouration remains unchanged in the absence of stress (i: no difference from time *i* to time *i+1*), while colouration is reduced in the presence of a stress factor (ii: colour reduction from time *i* to time *i+1*). In the presence of a stress factor animals activate the ELHS, thereby decreasing the negative effects of the stress. As a consequence, the colour reduction (ii) between time *i* and time *i+1* will be smaller when the PSR is stronger (iii, iv). If activated ELHS provokes the same effects in the presence and absence of stress factors, as suggested by several studies (Comendant et al. 2003; Meylan and Clobert 2005; Cote et al. 2006; Cabezas et al. 2007), activated ELHS may lead to increased colouration (v: colour increase from time *i* to time *i+1*).

cally associated with an increase in oxidative stress (Lin et al. 2004), suggesting that carotenoids may be used as antioxidants against oxidative stress and that corticosterone may reduce carotenoid-based colouration (through its effect on oxidative stress; Fig. 1 (ii)).

In contrast to acute stress, chronic stress has very different implications since it may change an animal's life history strategy from long-term to short-term investment (Dufty et al. 2002). Such a change may have potentially important consequences that include reproductive suppression (Sapolsky 1992), reduced immunocompetence (McEwen et al. 1997; Bartolomucci et al. 2005), decreased insulin production, neural degeneration (Bremner 1999) and reduced colouration (Fig. 1 (ii)). However, chronic stress produced by unpredictable adverse longer-term conditions (perturbation factors) such as drought, changes in social status, increased predator number, decreased food resources and diseases, is also responsible for animals entering an emergency life history stage (ELHS; Wingfield and Kitaysky 2002), whereby animals may try to reduce the negative effects of a stress. The ELHS theory may explain why in some species increased blood corticosterone levels due to chronic stress do not necessarily increase oxidative stress (Lin et al. 2006), and why a

blood corticosterone increase may even reduce oxidative stress during chronic stress (Hu et al. 2000). Hence, chronic stress is likely to affect colouration differently than acute stress. These findings are in line with studies showing that chronic corticosterone elevation positively affects survival (Comendant et al. 2003; Meylan and Clobert 2005; Cote et al. 2006; Cabezas et al. 2007). Indeed, they suggest that the negative effects of glucocorticosteroids may be compensated for (Fig. 1 (iii)), or even avoided (Fig. 1 (iv); Wingfield and Kitaysky 2002) if an animal is able to compensate the perturbing effects of a stress factor by activating the ELHS. These studies also suggest that successful compensation may depend on the presence of abundant food. Indeed, most studies providing evidence of the beneficial effects of chronic corticosterone exposure were carried out on well-fed individuals, potentially hiding the negative effects of elevated corticosterone concentrations (Breuner and Hahn 2003).

Disentangling the effects of a stress factor and the effects of the ELHS is not a simple task, since blood corticosterone levels are normally not increased and the ELHS is not activated in the absence of stress. Consequently, experimental designs are necessary that exclude the potentially negative effects of the stress factor and that activate the ELHS. For example, if parasite-induced stress provokes negative effects (Fig. 1 (iv)) the ELHS or the stress factor itself may be responsible for the negative outcome. In other words, if parasite abundance causes a negative effect and if the ELHS is the adaptive response to the stress, positive effects of ELHS may exist in the presence of abundant food (outcome (v) in Fig. 1), and negative effects in the absence of abundant food (outcome (ii)). However, if the ELHS is the cause of the negative effects, a negative change similar to outcome (ii) might occur, while outcome (i) would be expected if the ELHS were neutral.

Here, we investigated the effects of increased PSR on colouration in the presence of abundant food and thus, on the signal content of carotenoid-based colouration in the common lizard (*Lacerta vivipara*). First, we investigated the association between natural baseline corticosterone concentrations and colouration. Subsequently, we investigated the effects of corticosterone during chronic stress and its effects on carotenoid-based colouration on animals fed *ad libitum*. We predicted a negative colour change in corticosterone-treated lizards if increased blood corticosterone levels were to invariably lead to colour reduction (similar to Fig. 1 (ii)). If chronic corticosterone elevation activates the ELHS and the ELHS can compensate in the presence of abundant food, as suggested by some experimental studies (Comendant et al. 2003; Meylan and Clobert 2005; Cote et al. 2006; Cabezas et al. 2007; but see Calisi and Hews 2007), we predicted no colour change (Fig. 1 (i)) or even a positive colour change in corticosterone treated animals (Fig. (v)) due to the lack of an external stress.

Finally, we investigated the pigments responsible for the common lizard's colouration and tested whether common lizards are carotenoid limited. We also assessed whether limited carotenoid affects colouration by feeding half of the individuals in each corticoster-

one group with additional carotenoids. We predicted that lizards fed with carotenoids will have higher blood carotenoid levels and increased colouration when compared to the carotenoid limited group. This approach also allowed us to test whether a trade-off existed for carotenoids between colouration and the PSR (*i.e.*, if the PSR alters the carotenoid allocation through allostasis; Nelson 2005). If increased blood corticosterone levels were to have negative effects on colouration through their effect on carotenoid availability, we would predict that carotenoid limited lizards should show a colour reduction while carotenoid unrestricted lizards (the carotenoid supplemented animals) would show no or a smaller reduction in colour.

Materials and Methods

Species Description

The common lizard (*Lacerta vivipara*) is a small ovoviviparous Lacertidae that inhabits peat bogs and moist heathlands. The ventral colouration of males ranges from yellow to red with dark spots. In females, the ventral colouration ranges from cream to orange with only a few dark spots (Bauwens et al. 1987; Vercken et al. 2007). After birth, juveniles are melanic and they start developing their yellow-orange colouration during their first year of life. The fully developed colouration usually appears after the first or second hibernation (Vercken et al. 2007). Adult colouration is determined both genetically and environmentally (Vercken et al. 2007; Cote et al. 2008). During long-lasting stressful situations (*e.g.*, increased density), the colouration of the common lizard fades and becomes less red (Meylan et al. 2007; Cote et al. 2008). The function of ventral colouration has rarely been investigated in this species, although it has been shown experimentally that it plays an important role in courtship. Indeed, male common lizards that were painted with adult female colour patterns were courted like adult females (Bauwens et al. 1987; Vercken and Clobert 2008). More recent experiments show that ventral colouration plays an important role during sexual selection, given that females choose their mating partners based on this colouration (Fitze et al., submitted). The common lizard's yellow-orange ventral colouration is thought to stem from carotenoids (Czeczuga 1980). To confirm the basis of these colours and to investigate whether other pigments might also participate in producing the yellow-orange colouration (for example pteridines), we first analysed the pigments responsible for the ventral colouration. Thereafter, we investigated whether ventral colouration might be correlated with certain individual characteristics as seen in many other species.

Determination of colour pigments

Sample Collection.—To analyze which pigments were responsible for the yellow-orange belly colouration of the common lizard, we analyzed the skin of nine adult male common lizards originating from an experimental population at the Ecological Research Station of Foljuif (Seine-et-Marne, 48°17'N, 2°41'E). After capture, the males were decapitated and the ventral skin was detached from the muscles. Thereafter, the skin was weighed and individually stored in 1 mL acetone at –80 °C until it was analysed.

Carotenoid Extraction and HPLC Analyses.—Carotenoids were extracted from the skin, the acetone solution in which they were stored, and from plasma samples. The skin was washed in hexane, dried, weighed and homogenized in methanol in a Retsch MM 2000 micronizer at 27 Hz for 15 minutes (Hann, Germany) with ZrO containers. The residue was filtered (GHP Acrodisc 13 mm) and the methanol was evaporated (ThermoSavant SPD111V, New York, USA). The carotenoid residue was finally dissolved in 10 µL tetrahydrofuran (THF) and 90 µL of the mobile phase (70 : 30 acetonitrile : methanol), and it was immediately analyzed by High Performance Liquid Chromatography (HPLC, see below).

The sample (60 - 80 µL) was injected with an isocratic mobile phase onto a RP-18 column (YMC Europe GmbH, Schermbeck, Germany) fitted to a ThermoFinnigan (San Jose, USA) HPLC system with a PS4000 ternary pump, an AS3000 auto sampler, and an UV6000 diode-array UV/VIS detector. Chromatograms were obtained and analyzed with ChromQuest 4.0 software (ThermoFinnigan, San Jose, USA). The carotenoids were identified and quantified using lutein and zeaxanthin standards (Roche Vitamims Inc., Basel, Switzerland). Other carotenoids, such as astaxanthin, were compared (or matched) to previously run standards stored in the Chromquest library on the basis of the absorbance peak, lambda max and retention time. Carotenoids were only confirmed when they could be matched to the standards with a confidence limit of 99.9%. All concentrations were calculated as µg/g dry skin.

Measurement of baseline corticosterone levels in semi-natural and natural populations

Sample Collection.—We used blood samples from 256 adult lizards (68 females and 188 males) from natural (N = 69) and semi-natural populations (N = 187) to investigate the baseline corticosterone levels and their association with colouration. Individuals from natural populations were captured in May and June (2003 and 2004) from four different lizard populations on the Mont-Lozère in the Cévennes (1500 m a.s.l., Massif Central, Southeastern France, 44°00'N, 3° 45'E), while individuals from semi-natural populations at Foljuif were captured in May 2003. Apart from the standard measurements made on all lizards, spectroradiometric measurements were obtained from the lizards from the semi-natural populations. Blood samples were taken on all the lizards immediately after capture and they were conserved at –20 °C until they were analysed.

Corticosterone Measurement.—Blood corticosterone concentrations were determined using a single enzyme-immunoassay procedure (OCTEIA corticosterone kit, ref AC14F-1, lot 55642, IDS Inc, USA). In brief, this kit is a competitive enzyme-immunoassay utilizing a polyclonal antiserum against corticosterone coated onto the inner surface of polystyrene microtitre wells. Calibrators, controls and samples were incubated overnight at 28 °C with peroxidase-labelled corticosterone in the antibody-coated wells. The wells were then washed and a colour reaction was developed using the tetramethylbenzidine chromogen. The absorbance of the reaction mixtures

were read in a microplate reader, whereby the colour intensity developed was inversely proportional to the concentration of corticosterone in the samples. The blood corticosterone levels found were highly repeatable (correlation across individual repeats, $N = 4$; $F_{1,2} = 26.71$, $P = 0.036$, $r = 0.93$).

Effects of blood corticosterone levels and carotenoid availability on belly colouration

PRE-EXPERIMENTAL PROCEDURES.—Early in July 2002, we captured 88 adult males and 88 adult females from the four natural populations mentioned above. Lizards were moved to the laboratory at the Ecological Research Station at Foljuif (Seine-et-Marne, 48°17'N, 2°41'E) where they were individually housed in terrariums (terrarium size: 25 × 15 × 15 cm) under standard conditions (heat, light, water and food). Terrariums were heated on one side with a bulb (25 W) from 09:00 to 12:00 h and from 14:00 to 17:00 (for details see Fitze et al. 2005). The lizards were randomly distributed with respect to body size and population of origin within the laboratory (all $P > 0.6$). We measured the snout-vent length (SVL) and the tail length of each lizard to the nearest 1 mm. SVL is a determinant of mating success (Fitze et al. 2008; Fitze and Le Galliard 2008) and intra-sexual interactions (Fitze et al. 2008), and the tail length reflects energy storing capacity since lizards use the tail for fat storage (Chapple and Swain 2002). Body mass was measured to the nearest 0.002 g, and the colouration was measured as indicated below.

EXPERIMENTAL PROCEDURES.—The experiment started on August 2nd, 2002 (hereafter referred to as day one) when the lizards were randomly assigned to the different treatments. The body mass of each lizard was measured on the second and the 25th day of the experiment.

***Corticosterone application*:** We randomly assigned half of the lizards from each population and sex to a corticosterone group on day one (12 lizards per sex in three of the four populations and 8 lizards per sex in the fourth population), and the remaining lizards were assigned to a control group. The corticosterone treatment consisted of a daily application of 4.5 µL of sesame oil mixed with corticosterone (3 µg of corticosterone/µL oil; Meylan et al. 2003). Control lizards were treated with 4.5 µL of sesame oil alone (Cote et al. 2006; Vercken et al. 2007). The treatment was applied each evening to the lizards back to simulate chronic stress, commencing on the second day and ending on day 23 (Vercken et al. 2007).

***Carotenoid supplementation*:** On day one, half of the corticosterone treated and control treated lizards were assigned to a carotenoid supplementation group while the other half was assigned to a control group using a crossed two-factorial design. For each population and sex, the same number of lizards was attributed to one of the four treatment combinations. There were no significant differences at the start of the experiment in SVL, neither between carotenoid-treatment groups ($F_{1,172} = 0.053$, $P = 0.819$) nor between corticosterone-treatment groups ($F_{1,172} = 0.014$, $P = 0.905$), and the interaction between both was not significant ($F_{1,171} = 0.040$, $P = 0.841$). Similarly, there were no differ-

ences in the initial body condition among treatment groups (corticosterone- treatment: $F_{1,171} = 0.010$, $P = 0.919$; carotenoid-treatment: $F_{1,171} < 0.001$, $P = 0.992$; interaction: $F_{1,170} = 0.304$, $P = 0.582$). Lizards of the carotenoid supplementation group were administered the carotenoids lutein, zeaxanthin and β-carotene, the carotenoids that are naturally ingested when eating lepidopteran larvae (Partali et al. 1987). The carotenoid ratio (lutein: 77.4%, zeaxanthin: 6.1%, and β-carotene: 16.5% of total carotenoid content) with which lizards were fed was similar to that found in lepidopteran larvae (Partali et al. 1987). We diluted 818 mg lutein/zeaxanthin beadlets, containing 5.58% lutein and 0.44% zeaxanthin, and 130 mg β-carotene beadlets (containing 7.5% β-carotene) in 100 mL H_2O (total 0.5899 mg carotenoids /mL H_2O). Then, 0.03 mL of this solution (0.018 mg carotenoids) was injected with a sterile syringe into a moth larva (containing on average 1.6 µg/g carotenoids). The lizards were fed a single moth larva every five days and thus, carotenoid supplemented lizards ate between 0.018 mg and 0.106 mg carotenoids. Common lizards can eat more than nine moth larvae (average weight 200 mg) in 24 days and lepidopteran larvae, which are part of their natural diet, contain an average of 0.0033 mg carotenoids/g larvae (Partali et al. 1987). Thus, our carotenoid treatment corresponds to high doses ingested under natural conditions, which may compensate for the effects of the corticosterone treatment. Prior to supplementation, we injected the control moth larvae with 0.03 mL of a solution consisting of 948 mg control beadlets dissolved in 100 mL H_2O. Control beadlets contained of the same ingredients as the carotenoid beadlets except for the absence of carotenoids. All lizards were fed a single moth larva every five days, starting on the third day of the experiment and ending on the 23rd day of the experiment. Only larvae of similar body mass were used (254 mg ± 12.64 SE). Live larvae were presented to the lizards between 11:30 a.m. and 12:30 p.m and the lizards either accepted the food immediately (attacked and ate the larvae) or they refused it. In the latter case, we left the larva in the terrarium and checked in the evening whether the larva had been eaten. If not, it was removed from the terrarium. This procedure avoided confounding a lizard's appetite with a refusal due to human induced stress and it made sure that the exact number of larvae eaten during the entire experiment was determined.

Effects of corticosterone and carotenoid treatment on blood corticosterone and blood carotenoid concentrations

Effect of Corticosterone Application on Blood Corticosterone Concentrations.—Given that it was impossible to take blood samples without the risk of affecting the experimental outcome, we repeated the corticosterone treatment in 2006 using 36 males (captured from the same populations). This experiment enabled us to test the effects of the corticosterone treatment on blood corticosterone levels. Blood samples were taken at the end of the treatment and conserved at –20 °C until they were analysed.

Effect of Carotenoid Ingestion on Blood Carotenoid Concentration.—

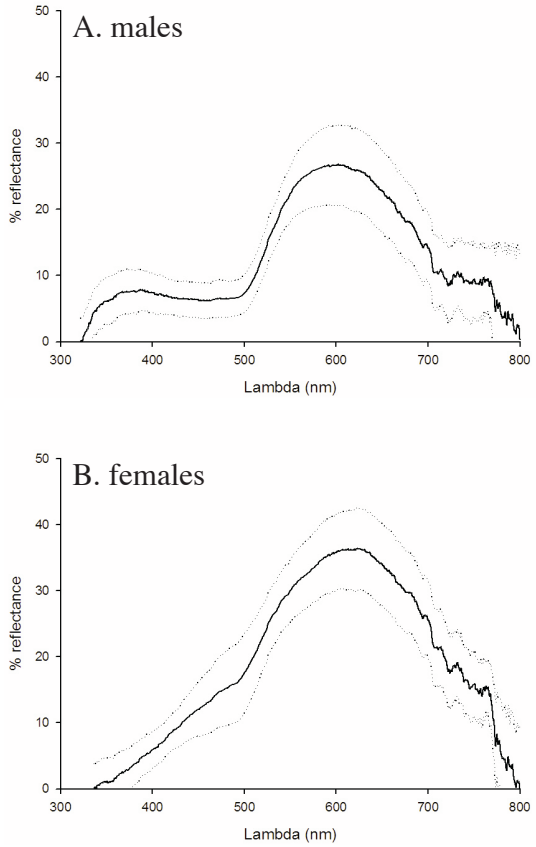

FIG. 2.—Average reflectance spectra of ventral colouration in male (**a)** and female (**b)** common lizards. The mean and SD per sex of the reflectance spectra measured at the beginning of the experiment are given.

To verify, whether the carotenoids ingested increased the blood carotenoid concentration, we conducted a carotenoid feeding experiment in 2007. Prior to the experiment we captured 14 common lizard males and transferred them to the lab. Seven randomly chosen male lizards were fed with carotenoids and another seven males were not given the carotenoid supplement according to the aforementioned protocol. After 20 days we took a blood sample (10 µL) that was centrifuged and stored at –80 °C until the blood carotenoids were analysed.

Colour measurement

Before instigating the experimental procedures and after they were terminated, the lizard's belly colouration was measured over the 300 nm to 700 nm visual spectrum at a 45 ° angle using a miniature spectroradiometer (USB2000, Ocean Optics Inc., Dunedin, FL, USA) and a Xenon light source (PX-2 and R400-7-UV/VIS, Ocean Optics Inc). Reflectance was measured in relation to a diffuse white standard (WS-1, Ocean Optics Inc.) uniformly reflecting 98 - 100% over the spectral range. For each lizard, we took a colour measurement on the breast, in the middle of the belly and on the anal plate, each corresponding to the average colouration of a surface of approximately 1mm^2, avoiding black spots. Average colouration per lizard was used for the analysis.

Since saturated carotenoid (and melanin) pigmentation removes most of the reflectance in the ultraviolet wavelengths (Fig. 2; Hill and McGraw 2006), we restricted the analyses to the human visible spectrum (400-700 nm). Using Endler's (1990) segment classification method, we derived objective estimates of hue (0-360°: 0° = red; 60° = yellow), chroma (0-100%), and brightness (0-100%), and we used the mean colouration of the three body parts measured in the analyses. The colour measurements taken three times from 218 lizards originating from the same populations were repeatable (Fitze and Le Galliard 2008) (hue: $F_{216,434} = 6.99$, $P < 0.0001$, $r = 0.66$; chroma: $F_{217,437} = 7.01$, $P < 0.0001$, $r = 0.67$;

brightness: $F_{217,437} = 10.5$, $P < 0.0001$, $r = 0.76$; Lessels and Boag 1987).

Statistics

To analyze whether specific traits were associated with the colouration we applied mixed model ANCOVAS. In these statistical models we included: population as a random factor, sex as a fixed factor, the covariates SVL, tail length and body mass, and the interactions with sex. The effects of carotenoid and corticosterone treatment on the change in the lizard's ventral colour (after minus before the experiment) were analyzed using mixed model ANCOVAS with sex, carotenoid and corticosterone treatment as fixed effects, population as a random effect, and food consumption and body mass change (final body mass – initial body mass) as covariates. For the analysis of the treatment effects on the body mass change, we applied ANOVA and for the effects on appetite, we applied a GLIMMIX with a Poisson error and a log link using SAS v8.2.0. (Littell et al. 1996). For the analyses of body condition, we simultaneously introduced body mass and SVL into the model. Therefore, body condition is a measure of the size-corrected body mass. For all the analyses, the full model included all the parameters and their interactions, and the final model was determined using backward elimination. The assumptions of the models applied were tested and they were met in all the analyses presented (*e.g.*, for parametric tests: equal variances among factor levels and normality of the residuals). All measurements were made blindly with respect to the treatments and the significance level was set at $P_{\text{two-tailed}} \leq 0.05$ for all tests. One control treated female died during the experiment and was therefore excluded from the analyses.

Results

Pigments responsible for the yellow-orange belly colouration

The yellow-orange colour disappeared from the skin within one hour when it was immersed in acetone and it adopted a bluish colour. This shows that the yellow-orange skin colour of common lizards stem from carotenoids and not from melanins or pteridins, neither of which are soluble in acetone (McGraw et al. 2004). In total, the skin contained 54.09 µg ± 4.67 SE carotenoids per gram tissue (5.144 µg ± 0.467 SE carotenoids per gram fresh weight), including lutein, zeaxanthin, astaxanthin, and canthaxanthin. Based on the dry weight of the skins (0.031 g ± 0.002 SE), we estimated that the yellow-orange belly colour derives from an absolute amount of 1.641 µg ± 0.149 SE of carotenoids. The skin's carotenoid concentration was negatively correlated with the lizard's hue ($t_{1,7} = 2.50$, $P = 0.041$, $r = -0.687$), which was measured prior to decapitation, and the skin's carotenoid concentration explained 47.2% of the variance in hue. There was no significant correlation between the lizard's chroma ($t_{1,7} = 1.45$, $P = 0.191$, $r = 0.480$) or the lizard's brightness ($F_{1,7} = 1.58$, $P = 0.159$, $r = 0.512$) and the lizard's skin carotenoid concentration. However, the sample size was small ($N = 9$) and thus, care must be taken in interpreting these results.

Table 1.—Effects of sex, body size and body mass on colouration of 175 common lizards caught in natural populations. The results from an ANCOVA analysis are shown with hue, chroma and brightness as dependent variables, sex as a fixed factor, population as a random factor, and SVL, tail length and body mass as covariates. The final model was determined using backward elimination and is shown in bold. Test statistics of backward eliminated variables are given before backward elimination.

	Hue		Chroma		Brightness	
Variable	test statistic	*P*	test statistic	*P*	test statistic	*P*
Sex	$F_{1,170} = 0.458$	0.499	**$F_{1,173} = 29.422$**	**<0.001**	**$F_{1,173} = 327.894$**	**<0.001**
SVL	$F_{1,171} = 0.802$	0.372	$F_{1,169} = 0.746$	0.389	$F_{1,172} = 1.595$	0.208
Tail length	$F_{1,172} = 2.872$	0.092	$F_{1,168} = 0.256$	0.614	$F_{1,171} = 0.869$	0.353
Body mass	**$F_{1,173} = 6.066$**	**0.015**	$F_{1,167} = 0.048$	0.828	$F_{1,170} = 0.024$	0.878
Population	$F_{3,167} < 0.001$	1.000	$F_{3,170} = 1.564$	0.200	$F_{3,167} < 0.001$	1.000

Belly colouration in relation to sex and phenotypic traits

Males ($N = 88$) had higher chroma (male: 0.325 ± 0.007 SE, female: 0.268 ± 0.008 SE) and lower brightness (male: 0.141 ± 0.003SE, female: 0.218 ± 0.003 SE) values than females ($N = 87$, Table 1), and there were no sex differences in the hue. There was a negative correlation between body mass (estimate: –3.905 ± 1.586 SE) and hue, indicating that individuals with greater body mass were redder in colour (Table 1). Tail length, body size and the population were not correlated with hue (Table 1). When co-linearity was assessed by including and excluding the different covariates in distinct orders, the final models were the same as those presented in Table 1. The quadratic terms of body size, tail length and body mass, and their interactions were not significant ($P > 0.1$).

Natural blood corticosterone levels

The mean basal blood corticosterone levels were 49.93 ± 39.14 ng/mL (range: 1 to 185 ng/mL). Body condition was positively correlated with the blood corticosterone levels ($F_{1, 253} = 15.902$, $P << 0.001$; 6.4% of the variance explained; estimate: 2.690 ± 0.603 SE [log(corticosterone level)^2 transformed data]), but this was not the case for body size ($F_{1, 251} = 0.875$, $P = 0.351$). There were no significant differences in blood corticosterone levels between sexes ($F_{1, 252} = 0.256$, $P = 0.613$). Lizards from experimental populations had lower blood corticosterone levels than lizards from natural populations ($F_{1,253} = 52.969$, $P << 0.001$; 17,04% of variance explained; estimate [experimental populations]: –2.898 ± 0.398 SE).

Hue was negatively correlated with the blood corticosterone levels (loglog transformed data: $F_{1, 185} = 11.357$, $P < 0.001$, estimate: –0.971 ± 0.288 SE; Fig. 3), while chroma was not correlated ($F_{1, 185} = 0.0003$, $P = 0.99$, estimate: 0.000 ± 0.000 SE). In addition, there was a trend for brightness to be

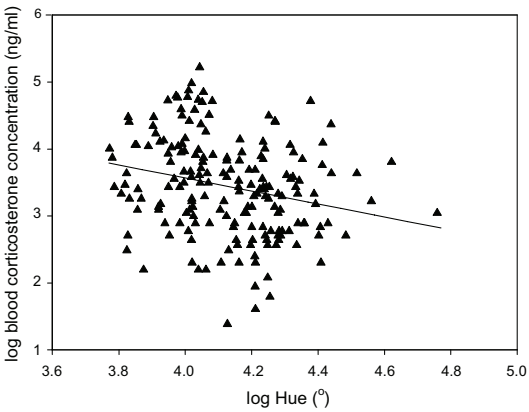

FIG. 3.—Correlation between basal blood corticosterone levels and the hue of the ventral colouration. The data were loglog transformed to meet the assumptions of the statistical analyses.

positively correlated with blood corticosterone levels ($F_{1, 185} = 3.59$, $P = 0.06$, estimate: 5.800 ± 3.061 SE).

Effects of corticosterone and carotenoids on blood content and belly colour

Blood corticosterone concentrations in corticosterone-treated males were significantly higher (138.23 ng/mL ± 16.86 SE) than those of placebo-treated males (86.73 ng/mL ± 15.93 SE: $F_{1,34} = 5.02$, $P = 0.03$, 25.75 ± 11.50 SE). Hence, the blood corticosterone concentrations following corticosterone administration were within the naturally occurring range (see paragraph on natural blood corticosterone levels).

During the experiment, the mean hue of the lizard's ventral colouration decreased from 71.6 ± 0.8 SE to 67.6 ± 0.7 SE, that is towards a redder colour (Fig. 4A, $P \leq 0.046$) and corticosterone treated lizards became significantly redder than control lizards (Table 2, Fig. 4A). The effect of corticosterone on hue was significant in all models of backward elimination, but it did not affect chroma or brightness (Table 2, Fig. 4B and 4C). There were no significant interactions between corticosterone treatment and any of the other parameters (all $P > 0.1$). Furthermore, sex, population, food consumption and change in

TABLE 2.—Effects of carotenoid supplementation and corticosterone treatment on colour change in hue, chroma and brightness. The results shown are of an ANCOVA analysis with: hue, chroma and brightness as dependent variables; sex as a fixed factor; population as a random factor; and the number of larvae eaten and the body mass change as covariates. The final model was determined using backward elimination and is shown in bold. Test statistics of backward eliminated variables are given before backward elimination.

	Hue		Chroma		Brightness	
Variable	test statistic	*P*	test statistic	*P*	test statistic	*P*
Corticosterone	**$F_{1,168} = 8.549$**	**0.004**	$F_{1,161} = 0.030$	0.863	$F_{1,161} < 0.01$	0.989
Carotenoid	$F_{1,164} < 0.01$	0.981	$F_{1,162} = 0.225$	0.636	$F_{1,168} = 1.588$	0.209
Sex	$F_{1,167} = 1.210$	0.273	$F_{1,166} = 0.600$	0.440	$F_{1,165} = 0.200$	0.655
Number larvae eaten	$F_{1,166} = 2.430$	0.121	$F_{1,167} = 2.986$	0.086	$F_{1,166} = 0.183$	0.669
Body mass change	$F_{1,165} = 0.157$	0.693	$F_{1,168} = 0.779$	0.379	$F_{1,167} = 1.735$	0.190
Population	$F_{3,161} < 0.01$	0.981	$F_{3,163} = 0.901$	0.442	$F_{3,162} = 0.074$	0.974

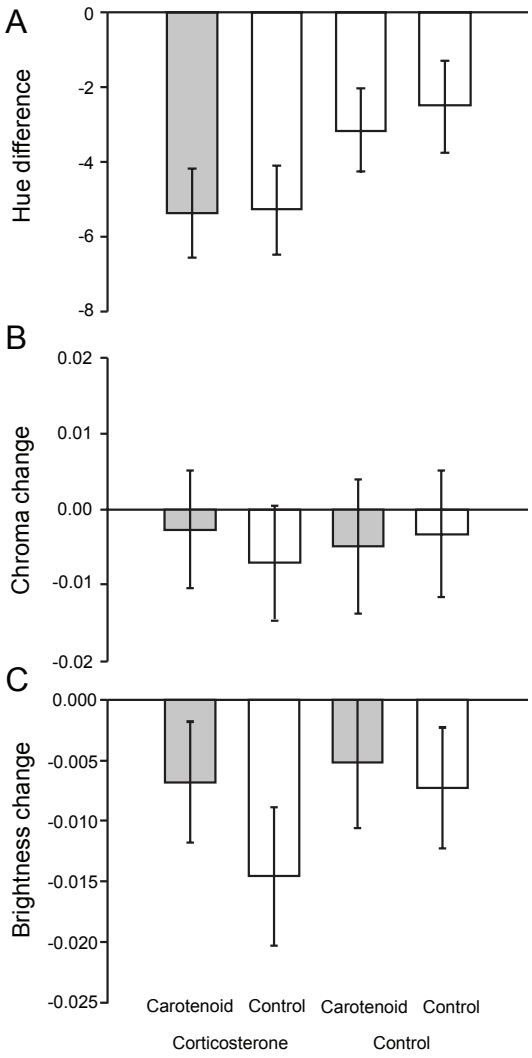

FIG. 4.—Change of the lizard's belly colouration in (**a)** hue, (**b)** chroma and (**c)** brightness in relation to carotenoid and corticosterone treatment. Raw means (± SE) of the colour change (colour after treatment – colour before treatment) are shown and Table 1 shows the statistical analysis.

body mass did not significantly affect the colour change. Carotenoid supplementation led to an increase in blood carotenoid concentration (lutein: $F_{1,12}$ = 17.567, P = 0.001, estimate = 7.526 ± 1.796 µg/mL, R^2 = 0.59; zeaxanthin: $F_{1,12}$ = 12.443, P = 0.004, estimate = 0.47 ± 0.133, R^2 = 0.51), but it did not affect ventral colouration (Table 2, Fig. 4A-C). Moreover, there were no significant interactions between carotenoid treatment and the other parameters (all $P > 0.1$). The changes in both chroma and brightness of all treatment groups were not different from zero (Fig. 4B, C, all $P \geq 0.16$). Over the course of the experiment, lizards ate an average of 3.989 larvae ± 0.0 SE and thus the carotenoid fed lizards consumed an average of 0.071 mg ± 0.002 SE carotenoids. The average amount of carotenoids ingested corresponded to 43 times the amount of carotenoids responsible for the ventral colouration, and around 4 times the amount of carotenoids present in the skin, intestine and liver (Czeczuga 1980; Gavaud 1986).

Effects of corticosterone and carotenoids on body mass and appetite.

Corticosterone-treated lizards had enhanced appetite (corticosterone treated: 3.44 ± 0.04, control: 3.12 ± 0.05 larvae eaten, $F_{1,172}$ = 5.95, P = 0.016), and there were no differences in appetite between carotenoid-fed and control-fed lizards ($F_{1,157}$ = 0.06, P = 0.805; estimate: –0.010 ± 0.040 SE). The interaction between carotenoid and corticosterone treatment was not significant ($F_{1,137} < 0.01$, P = 0.958) and likewise, population and all other interactions did not significantly affect appetite ($P > 0.2$). Females ate more than males ($F_{1,172}$ = 33.28, $P < 0.001$, estimate: 0.235 ± 0.041 SE) and as such, the females increased their body mass while the body mass of the males remained almost constant ($F_{1,173}$ = 27.66, $P < 0.001$, estimate: 0.055 ± 0.010

SE). The change in body mass did not differ between corticosterone and control administered ($F_{1,172}$ = 0.51, P = 0.478, estimate: –0.007 ± 0.010 SE) or carotenoid and control fed animals ($F_{1,171}$ = 0.39, P = 0.533, estimate: 0.007 ± 0.001 SE), and the interaction between carotenoid and corticosterone treatment was not significant ($F_{1,160}$ = 0.51, P = 0.476). There were no significant differences between populations and none of the interactions were significant (P > 0.05).

Discussion

We have investigated the effects of carotenoid availability and blood corticosterone levels on the sexually selected ventral colouration of the common lizard. We first determined the chemical basis of the lizard's bright yellow-orange belly colour. The results indicate that carotenoids are responsible for the colouration. Melanins and pteridins do not appear to contribute to the yellow-orange colour, since no yellow-orange pigments remained in the skin after acetone extraction (McGraw et al. 2004). The carotenoid-content of the skin was negatively correlated with the hue of the lizard's skin colouration, explaining around 47% of the observed variation. This indicates that measuring skin colouration enables its carotenoid-content to be assessed.

Carotenoid supplementation did not induce any change in colour, although it did lead to an increase in blood carotenoid concentrations. Since the power to find an effect on colouration of similar size (d) as that observed in Tschirren *et al.* (2003) (d = 1.073; δ = 7.1) or in Fitze *et al.* (2003) (d = 0.841; δ = 5.6) was 100%, our results imply that carotenoid feeding does not affect carotenoid-based colouration in this species, in contrast to studies on birds and fish (Milinski and Bakker 1990; Blount et al. 2003; Tschirren et al. 2003; Hill 1992). This finding is in line with the existence of colour morphs (Sinervo et al. 2007) and together with an earlier study, that estimated the heritability of the common lizard's colouration at 0.5 (Vercken et al. 2007), it suggests that the three types of colour morphs described in the common lizard (white, yellow, and orange) may be the result of differential carotenoid-incorporation, potentially explaining how colouration may reveal an individual's life-history strategy (Sinervo et al. 2001; Sinervo and Clobert 2003).

It has been shown that colour signals are usually genetically and environmentally determined (Kodric-Brown 1989; Tschirren et al. 2003; Fitze et al. 2003; Hill and McGraw 2006; Fitze et al. 2007). Environmental determination of the common lizard's colouration has already been proposed (Cote et al. 2008) and our results show that ventral colouration varies with environmental conditions (*i.e.*, stress response). Chronically increased blood corticosterone levels negatively affected the hue of the ventral colour in both male and female lizards (shifting towards a redder colouration). Similarly, increased blood corticosterone levels were associated with redder colouration in non-manipulated lizards from natural populations (Fig. 3). This indicates that increases in corticosterone, also found during PSR, are not directly responsible for the colour fading observed during stressful situations in the common lizard (Meylan et al.

2007; Cote et al. 2008). The effect of corticosterone on colouration may have been the result of enhanced appetite, potentially leading to increased carotenoid transport and hence, to an increase in colouration. However, the observed colour change in corticosterone-treated individuals was not explained by carotenoid-availability, food consumption or a change in body condition, indicating that the effect of corticosterone was not due to differential food intake. Corticosterone may also provoke a reallocation of carotenoids (*e.g.*, from the liver to the blood), since pigmentation is often under hormonal control (such as androgens or oestrogens; Hill and McGraw 2006). Such reallocation may occur through the hormones' effects on carotenoid transporting proteins (Servatius et al. 1994) or through direct reallocation of carotenoids from storage sites to the blood stream (McGraw et al. 2006), both resulting in changes of the blood carotenoid concentrations. Since increased concentrations of blood carotenoids did not affect colouration, it is unlikely that the effect of corticosterone on colouration was mediated by carotenoids. Hence, the mechanism by which corticosterone affects carotenoid-based colouration of common lizards remains unknown and investigating these mechanisms should be addressed in future studies.

Whatever the precise physiological mechanism, the results presented here are in line with the ELHS theory, suggesting that chronically enhanced corticosterone levels may activate the ELHS, and given that food was abundant this may have led to positive rather than negative effects (Fig. 1 (v); see also Hu et al. 2000). Alternatively, chronic increases in blood corticosterone levels may have changed an animal's life history strategy (Dufty et al. 2002) from long-term to short-term investment. As a consequence, lizards may invest mainly in short-term survival, as shown previously (Comendant et al. 2003; Meylan and Clobert 2005; Cote et al. 2006; Cabezas et al. 2007), and in sexual attractiveness by becoming redder. Indeed, in experiments where adult males had the opportunity to copulate with three different females, redder males (smaller hue values) copulated with more females (ANOVA: $F_{2,38} = 3.535$, $P = 0.039$; not copulating: 69.592° ± 1.182 SE, once copulating: 67.965° ± 1.841 SE, twice copulating: 58.059° ± 2.817 SE; for experimental design see Fitze et al. 2008), suggesting that lizards may have made a kind of 'terminal investment' (Cluttonbrock 1984). These two scenarios are not incompatible since the ELHS might be the physiological mechanism by which the strategy "terminal investment" is activated. However, future studies will be necessary to reveal whether increased corticosterone levels truly have positive effects under circumstances of food restriction.

Our study clearly shows that the ventral colouration of common lizards is partly determined environmentally and it suggests that belly colouration may be a condition-dependent trait. Condition-dependency is in agreement with studies on the colouration of other lizards (Calisi and Hews 2007) and it is in line with studies on birds and fish, where carotenoid-based colouration is a condition-dependent trait that honestly reflects an individual's quality (Hill 1991; Blount et al. 2003; Fitze et al. 2003; Tschirren et al. 2003). How-

ever, our study also suggests that unlike in birds and fish, the condition-dependency may not be due to carotenoid availability. Since carotenoid availability did not affect colouration, the common lizard's belly colour may be a composite trait, with genetic determination of the carotenoid-based element and condition-dependent determination of yet unidentified components, like cell structure, melanophore content or iridophore content (Cooper and Greenberg 1992). Consequently, females who select redder males are selecting males with increased stress responses but not males with higher carotenoid-availability.

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Chapter III

Vitamin E, Vitamin A, and Carotenoids in Male Common Lizard Tissues

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Abstract: Vitamin E, vitamin A, and carotenoids are essential micronutrients for animals because of their antioxidant and immunostimulant functions and their implications for growth, development, and reproduction. In contrast to mammals and birds, information about their occurrence and distribution is generally lacking in reptiles, constraining our understanding of the use of these micronutrients. Using high-performance liquid chromatography (HPLC), we determined the concentrations of vitamin E, vitamin A, and carotenoids in plasma, storage sites (liver and abdominal fat bodies), and in the colored ventral skin of male common lizards, *Lacerta vivipara*. All tissues shared a similar micronutrient profile, except the liver, which also showed traces of vitamin A_1. The main vitamin E compound present was α-tocopherol followed by lower concentrations of γ-(β-) tocopherol. Vitamin A_2 was the main Vitamin A compound and it showed the highest concentration in the liver, where vitamin A_2 esters and traces of vitamin A_1 were found. Lutein was the main carotenoid and it formed esters in the liver and the ventral skin. Zeaxanthin and low concentrations of β-carotene were also present. The liver was the main storage site for carotenoids and vitamin A whereas hepatic vitamin E concentrations resembled those present in abdominal fat bodies. Compared with abdominal fat bodies, the ventral skin contained lower concentrations of vitamin A and vitamin E, but similar concentrations of carotenoids. These results suggest that important differences exist in micronutrient presence, concentration, and distribution among tissues of lizards and other taxa such as birds and mammals.

Key words: Coloration; Dehydroretinol; Nutrition; Oxidative stress; Retinol; Squamata; Vitamin A metabolism; Xanthophylls.

Vitamina E, Vitamina A y Carotenoides en Tejidos de Machos de Lagartija de Turbera

Resumen: La vitamina E, la vitamina A y los carotenoides son micronutrientes esenciales para los animales debido a sus funciones antioxidantes e inmunoestimulantes así como a sus implicaciones para el crecimiento, desarrollo y reproducción. Al contrario que en mamíferos y aves, la información acerca de su presencia y distribución en reptiles es escasa, lo que, en último término, limita el conocimiento acerca de la utilización de estos micronutrientes. Mediante cromatografía líquida de alta resolución, se determinaron las concentraciones de vitamina E, vitamina A y carotenoides en el plasma, órganos de reserva (hígado y cuerpos grasos abdominales) y en la coloreada piel ventral de varios machos de lagartija de turbera, *Lacerta vivipara*. Todos los tejidos presentaron un perfil similar de micronutrientes a excepción del hígado donde se encontraron trazas de vitamina A_1. La principal forma de vitamina E encontrada fue α-tocoferol seguido por menores concentraciones de γ-(β-) tocoferol. La vitamina A_2 fue la principal forma de vitamina A. Su mayor concentración se encontró en el hígado donde también se encontraron ésteres de vitamina A_2 y trazas de vitamina A_1. La luteina fue el carotenoide predominante, parte de la cual se encontró en forma de ésteres en el hígado y en la piel ventral. También se detectó zeaxantina y bajas concentraciones de β-caroteno. El hígado fue el principal órgano de reserva de carotenoides y de vitamina A, mientras que las concentraciones hepáticas de vitamina E fueron similares en este órgano a las presentes en los cuerpos grasos del abdomen. En comparación con estos últimos, la piel del vientre presentó menores concentraciones tanto de vitamina A como de vitamina E aunque similares concentraciones de carotenoides. Estos resultados sugieren que existen diferencias importantes en la presencia, concentración y distribución de micronutrientes entre los tejidos de lagartijas y los de otros taxones tales como aves y mamíferos.

Introduction

Vitamin E, vitamin A, and carotenoids are essential micronutrients required by animals for metabolic, immunological, reproductive, and developmental processes (Ames 2006). Animals are not able to synthesize them *de novo* and thus depend on dietary intake to meet their basic nutritional requirements (Surai 2002). Vitamin E (*i.e.*, isomeric forms of tocopherol and tocotrienol) mainly functions as a potent antioxidant (Burton and Traber 1990; Rock et al. 1996). Vitamin A compounds (retinoids that exhibit retinol activity; Blomhoff et al. 1990) enhance cellular proliferation and differentiation (McCaffery et al. 2003; Mora et al. 2008), promote embryonic growth and development and postnatal bone remodeling (Edem 2009), and constitute the chromophore of visual pigments in the retina (Pepe 1999). In animals, carotenoids serve as ornamental pigments (Olson and Owens 1998), antioxidants, immune enhancers, and vitamin A precursors (Simpson 1983; Olson 1993; Surai 2002; Chew and Park 2004).

Current knowledge about the occurrence and implications of vitamin A, vitamin E, and carotenoids mostly comes from studies on humans, other mammals, and birds, whereas limited information is available in reptiles, particularly in squamates. This is especially surprising given that deficiency of these micronutrients impairs growth, reproduction, and survival and provokes skin, respiratory, and ocular diseases, both in captive (Boyer 1996; Ferguson et al. 1996; Goodman 2007) and free-ranging reptiles (Holladay et al. 2001; Brown et al. 2004). These micronutrients may play an important role in reptile behavior, for example through femoral gland secretions and ornamental coloration (Steffen and McGraw 2007; Fitze et al. 2009; Martín and López 2010). Despite their importance, research mainly focused on egg and less importantly on blood (Thompson et al. 1999a; Thompson et al. 1999b; Thompson et al. 1999c; Dierenfeld et al. 2002) and studies investigating micronutrient content in other tissues are lacking.

In this study, we determined the profile and concentrations of vitamin A, vitamin E, and carotenoids present in male common lizards, *Lacerta vivipara*. *Lacerta vivipara* is a small ground-dwelling lacertid lizard that inhabits peat bogs and moist heathlands. Its distribution ranges from northern Spain and Greece at the southern boundary up to northern Europe and Asia. It inhabits mainly cold climates with overwintering sites exposed to subzero temperatures (Sindaco and Jeremcenko 2008). It feeds on small invertebrates, mostly spiders and Homoptera (Avery 1966; Heulin 1986) and also on larger prey like earthworms (L. M. San-Jose, personal observation). In the Pyrenean populations, where the study was conducted, adult *L. vivipara* males develop a conspicuous ventral coloration that ranges from white to yellow and orange (Sinervo et al. 2007). Coloration spreads from the base of the tail to the collar scales and rarely reaches throat scales. Yellow-orange ornamental pigmentation stems from carotenoids, which have also been detected in other tissues of *L. vivipara* (Czeczuga 1980; Fitze et al. 2009). Nothing is known about the presence and content of vitamin A and vitamin E in this species, with the exception of α-

tocopherol, which is present in femoral gland secretions (Gabirot et al. 2008). Using high-performance liquid chromatography (HPLC) analyses, we investigated the content of vitamin A, vitamin E, and carotenoids present in plasma, main reserve organs (liver and abdominal fat bodies), and ventral skin of male Common Lizards. In particular, we tested in each tissue whether different vitamin E compounds showed different concentrations and, similarly, whether different carotenoid species showed different concentrations. For each vitamin E and vitamin A compound, and for each carotenoid species, we also tested whether the concentration differed among liver, ventral skin, and abdominal fat bodies.

Materials and Methods

Lizard Handling

In June 2008, we captured eight adult male common lizards (snout-to-vent length [SVL] range: 50–56 mm) at Somport (Central Pyrenees, Huesca, Spain, 42°47'N, 0°31'W; datum = European Datum 1950). They were brought to the laboratory at the Instituto Pirenaico de Ecología (Jaca, Huesca, Spain), where we measured SVL (to the nearest 1 mm) and body mass (to the nearest 0.001 g). Lizards were individually housed in plastic terraria (25 × 15 × 15 cm) equipped with two shelters, a water pond, and peat bog as substrate. A 40-W bulb placed in a corner of each terrarium provided light and heat following a 10-h light:14-h dark photoperiod and an ultraviolet (UV) light source provided UVB and UVA for 2 h per day (Fitze et al. 2008, 2010). Lizards were fed every 2 d with one wax worm larva, *Galleria mellonella*, and water was provided *ad libitum*. Lizards were captured, housed, and decapitated according to the permits provided by the Gobierno de Aragón, Spain (see Acknowledgments for permit numbers).

Tissue Sampling

Twenty days after capture, a blood sample was collected from the retro-orbital sinus of each lizard using a heparinized microcapillary tube. An average (± SE) of 9.81 µL (± 2.04) of plasma was obtained after blood centrifugation (5 min at 8900 g). After blood sampling, lizards were anesthetized by subcutaneously injecting 0.02 mL/g of a metomidine and ketamine solution (1:50) and thereafter decapitated. Abdominal fat bodies, liver, and the ventral skin between collar scales and anal plate were harvested. All tissues were rinsed twice with phosphate-buffered saline to avoid contamination with the remaining blood and weighed to the nearest 0.01 mg. On average (± SE) 37.76 mg (± 6.55) of abdominal fat bodies, 98.05 mg (± 6.46) of liver, and 87.03 mg (± 4.78) of ventral skin were obtained and used for subsequent analyses. All samples were stored for 2 mo at –20°C and then moved to –80°C until HPLC analyses (*ca.* 1 mo).

HPLC Analyses

Vitamin E, vitamin A, and carotenoids of abdominal fat bodies, liver, ventral skin, and blood plasma were analyzed by HPLC using an adapted protocol of Olmedilla et al. (1997). Plasma samples were mixed with 0.1 mL of distilled water and 0.2 mL of ethanol, vortexed, and extracted twice with 0.5 mL of methylene chloride–hexane (1:5). Organic phases were pooled, evaporated to dryness,

reconstituted in tetrahydrofuran (THF)–ethanol (EtOH), and injected onto the HPLC column. Abdominal fat bodies, liver, and ventral skin samples were placed in EtOH for 25 min. Extraction was performed by ultrasound and intermittent vortex for 5 min. Water (1 mL) and 2 mL of methylene chloride–hexane (1:5) were added, vortexed, centrifuged, pooled, evaporated to dryness, reconstituted (THF–EtOH), and injected onto the HPLC column.

A subset of five liver and four ventral skin samples were analyzed twice (once saponified and once not saponified) and their HPLC chromatograms were compared to evaluate the presence of vitamin A and carotenoid esters. Ester forms show the same absorption spectra but longer retention times than free forms (Ladislav et al. 2005) and thus chromatograms where esters are present have more peaks than chromatograms where only free forms are present. Saponification results in ester hydrolysis, leading to the presence of free forms only. Consequently, chromatograms of saponified samples show fewer peaks and bigger absorption peaks of free forms compared with chromatograms of not saponified samples. Tissue samples were saponified with methanolic potassium hydroxide following the protocol of Granado-Lorencio et al. (2001). First, reconstituted extracts were evaporated and reconstituted with pyrogallic acid (0.3 M) dissolved in ethanol. Saturated methanolic potassium hydroxide was added and the mixture was vortexed for 3–5 min. Partition was carried out by adding distilled water and methylene chloride–hexane. Finally, the organic phases were pooled, evaporated to dryness, reconstituted (THF–EtOH) and injected onto the HPLC column.

The chromatographic system consisted of a Spheri-5-ODS column (Applied Biosystem, San Jose, CA) with gradient elution of acetonitrile–methanol (85:15) for 5 min to acetonitrile–methylene chloride–methanol (70:20:10) for 20 min. The injection volume was 10 µL and the flow rate was 1.8 mL/min. Ammonium acetate (0.025 M) was added to the methanol. Isomeric forms of tocopherol (vitamin E) were detected using a photodiode array detector (model 2996, Waters Associates, Milford, MA) set at 295 nm and fluorescence (excitation: 290 nm, emission: 330 nm). Under these assay conditions γ- and β-tocopherol (hereafter referred as γ-(β-) tocopherol) could not be distinguished and δ-tocopherol concentration could not be determined. By setting the photodiode array detector at 326 nm we could detect dehydroretinol (vitamin A_2 alcohol) and retinol (vitamin A_1 alcohol). Concentrations of vitamin A_2 were measured as relative response (absorbance units full scale per milligram tissue). Carotenoid detection was carried out by setting the photodiode array at 450 nm. Using this method, *trans*-lutein, zeaxanthin, 13/15-*cis*-lutein, α-carotene, all-*trans*-β-carotene, 9-*cis*-β-carotene, and 13/15-*cis*-β-carotene, among other carotenoids, could be determined simultaneously. Compound identification was carried out by comparing retention times of the samples with those of authentic standards and with online UV-visible spectra. The short- and long-term precision and accuracy of the analytical method were verified periodically through participation in the Fat-Soluble Qual-

ity Assurance Program conducted by the National Institute of Standards and Technology (Gaithersburg, MD).

Statistics

For each tissue, we tested whether different vitamin E compounds showed different concentrations using Wilcoxon signed-rank tests for small sample sizes. We also tested for each tissue whether different carotenoid species showed different concentrations using Friedman tests and Wilcoxon signed-rank tests for *post hoc* comparisons.

To test whether compound concentrations differed among liver, ventral skin, and abdominal fat bodies we used Friedman tests. Wilcoxon signed-rank tests were used for *post hoc* comparisons between pairs of tissues. Quantitative comparisons between plasma and other tissues were not possible because plasma concentrations were volume referenced and tissue samples were mass referenced.

All the statistical analyses were conducted using R v 2.10.1 software (Free Software Foundation, GNU Project, Boston, MA). For all statistical models the significance level was set at $\alpha = 0.05$ (two-tailed) and adjusted for *post hoc* comparisons using sequential Bonferroni procedures (Hochberg 1988).

Results

Vitamin E

In all samples, vitamin E compounds α-, γ-(β-), and δ-tocopherol were detected. α-Tocopherol was the predominant compound showing significantly higher concentrations than γ-(β-) tocopherol in plasma ($W = 36$, $N = 8$, $P = 0.008$), liver ($W = 36$, $N = 8$, $P = 0.008$), fat bodies ($W = 36$, $N = 8$, $P = 0.008$), and ventral skin ($W = 36$, $N = 8$, $P = 0.008$; Table 1). There existed significant differences between liver, fat bodies, and ventral skin in the concentrations of α-tocopherol (Friedman's $\chi^2 = 9.75$, d.f.: 2, $P = 0.008$) and γ-(β-) tocopherol (Friedman's $\chi^2 = 13$, d.f.: 2, $P = 0.002$). *Post hoc* analyses revealed that both α-tocopherol and γ-(β-) tocopherol were less concentrated in the skin than in the liver and fat bodies (Table 1).

Vitamin A

In all samples, vitamin A was mainly present as dehydroretinol (vitamin A_2) and traces (< 0.001 µg/mg) of retinol (vitamin A_1) were only found in five liver samples (Table 1). Our analyses therefore concentrate on vitamin A_2. There existed significant differences in vitamin A_2 concentrations among tissues (Friedman's $\chi^2 = 16$, d.f.: 2, $P < 0.001$). The highest and lowest concentrations were found in the liver and in the ventral skin respectively, whereas fat bodies showed an intermediate concentration (Table 1). Esters of vitamin A_2 were detected in the liver but not in the other tissues (Fig. 1).

Carotenoids

Lutein and zeaxanthin were the most abundant carotenoids in all tissue samples (mean ± SE; 78.9 ± 0.1 % and 20.2 ± 0.1 % of the total carotenoid concentration, respectively; Table 1). β-Carotene (mean ± SE; 1.2 ± 0.1 %) was found in the liver, skin, and fat bodies of all individuals, but only in one individual did we detect β-carotene in the plasma (0.04 µg/mL). Lutein esters were identified in

TABLE 1.—Concentration (mean ± SE) of vitamin E, vitamin A, and carotenoids measured in plasma, liver, abdominal fat bodies, and ventral skin of eight male common lizards (*Lacerta vivipara*) and results from *post hoc* (Wilcoxon signed-rank) tests among liver, fat bodies, and ventral skin.

Micronutrients	Lizard samples				Comparisons		
	Plasma	Liver	Abdominal fat bodies	Ventral skin	Liver *vs.* abdominal fat bodies	Liver *vs.* ventral skin	Abdominal fat bodies *vs.* ventral skin
Vitamin E							
α-Tocopherol	22.299 ± 2.548 (μg/mL)	2.833 ± 0.255 (μg/mg)	2.956 ± 0.714 (μg/mg)	0.807 ± 0.108 (μg/mg)	$W = 15$, $P = 0.742$	$W = 36$, $P = 0.023$	$W = 35$, $P = 0.031$
γ-(β-) Tocopherol	8.286 ± 1.134 (μg/mL)	0.832 ± 0.073 (μg/mg)	1.112 ± 0.150 (μg/mg)	0.205 ± 0.015 (μg/mg)	$W = 28$, $P = 0.195$	$W = 36$, $P = 0.016$	$W = 36$, $P = 0.016$
Vitamin A							
Vitamin A_1	—	< 0.001 (μg/mg)	—	—			
Vitamin A_2	232.9 ± 34.7 (AUFS/μL)	985.0 ± 25.6 (AUFS/mg)	163.9 ± 26.94 (AUFS/mg)	90.70 ± 9.31 (AUFS/mg)	$W = 36$, $P = 0.008$	$W = 36$, $P = 0.008$	$W = 36$, $P = 0.008$
Carotenoids							
Lutein	4.928 ± 0.566 (μg/mL)	8.535 ± 2.225 (μg/mg)	0.153 ± 0.055 (μg/mg)	0.218 ± 0.019 (μg/mg)	$W = 36$, $P = 0.016$	$W = 36$, $P = 0.016$	$W = 26$, $P = 0.313$
Zeaxanthin	1.357 ± 0.128 (μg/mL)	1.436 ± 0.283 (μg/mg)	0.046 ± 0.016 (μg/mg)	0.054 ± 0.004 (μg/mg)	$W = 36$, $P = 0.016$	$W = 36$, $P = 0.016$	$W = 24$, $P = 0.461$
β-Carotene	—	0.039 ± 0.010 (μg/mg)	0.005 ± 0.003 (μg/mg)	0.003 ± 0.001 (μg/mg)	$W = 36$, $P = 0.016$	$W = 36$, $P = 0.016$	$W = 16$, $P = 0.844$

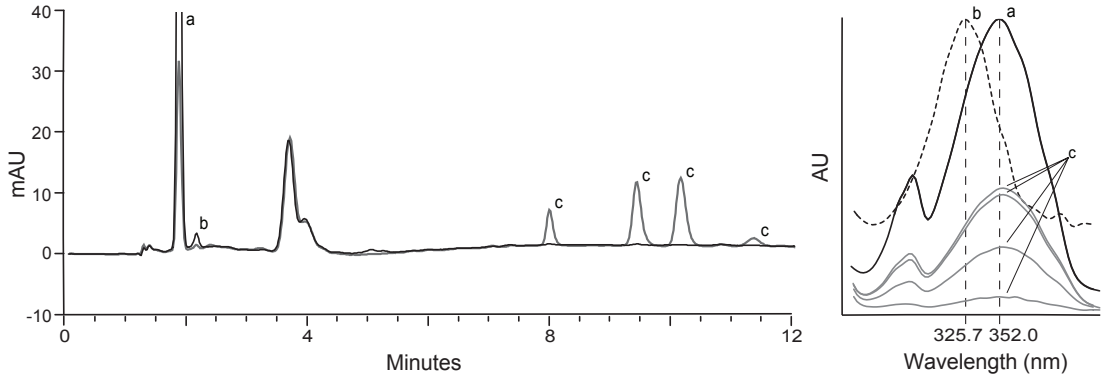

FIG. 1.—High-performance liquid chromatography chromatogram (at 326 nm and in miliabsorbance units, mAU) and absorption spectra (in absorbance units, AU) of vitamin A compounds (a, vitamin A_2 alcohol; b, vitamin A_1 alcohol;c, vitamin A_2 esters) present in the liver of male common lizards (*Lacerta vivipara*) before (gray line) and after (dark line) hydrolysis.

ventral skin and liver samples. After hydrolysis, lutein ester peaks disappeared from the chromatograms, resulting in increased lutein concentrations (Fig. 2). Zeaxanthin concentration also increased after hydrolysis, suggesting that either zeaxanthin esters or lutein isomers (*e.g.*, 9-*cis*-lutein coeluting with zeaxanthin) might exist at low concentrations.

In plasma, lutein was significantly more concentrated than zeaxanthin ($W = 36$, $N = 8$, $P = 0.008$). There also existed differences among lutein, zeaxanthin, and β-carotene concentrations of liver (Friedman's $\chi^2 = 16$, d.f.: 2, $P < 0.001$), fat bodies (Friedman's $\chi^2 = 16$, d.f.: 2, $P < 0.001$), and ventral skin (Friedman's $\chi^2 = 16$, d.f.: 2, $P < 0.001$). In these tissues, lutein concentrations were significantly higher than zeaxanthin and β-carotene concentrations and zeaxanthin concentrations were significantly higher than β-carotene concentrations (for all Wilcoxon *post hoc* tests $W = 36$, $N = 8$, $P = 0.008$). The concentration of lutein, zeaxanthin, and β-carotene significantly differed among liver, fat bodies, and ventral skin (lutein: Friedman's $\chi^2 = 13$, d.f.: 2, $P = 0.002$, zeaxanthin: Friedman's $\chi^2 = 12.25$, d.f.: 2, $P = 0.002$, β-carotene: Friedman's $\chi^2 = 12.25$, d.f.: 2, $P = 0.002$). Fat and ventral skin concentrations of lutein, zeaxanthin, and β-carotene were lower than liver concentrations (Table 1). Lutein concentration tended to be higher in the ventral skin than in fat bodies and there existed no significant differences between fat and ventral skin concentrations of zeaxanthin and β-carotene.

DISCUSSION

Vitamin E

α-Tocopherol was the predominant vitamin E compound in all tissues of *L. vivipara* males. Its constant predominance together with the presence of γ-(β-) tocopherol in all analyzed tissues may indicate that *L.* vivipara males do not selectively absorb or store different vitamin E compounds, as previously observed in birds and mammals (Traber and Sies

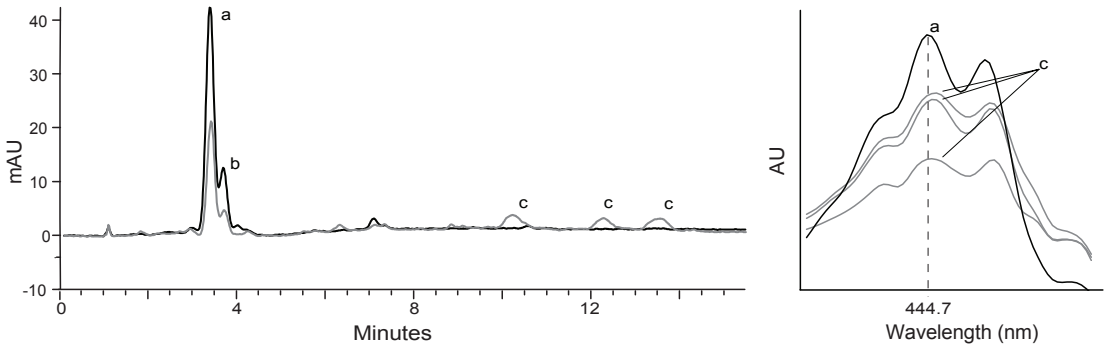

FIG. 2.—High-performance liquid chromatography chromatogram (at 450 nm and in miliabsorbance units, mAU) and absorption spectra (in absorbance units, AU) of different carotenoids (a, lutein; b, zeaxanthin; c, lutein esters) present in the ornamented ventral skin of the male common lizards (*Lacerta vivipara*) before (gray line) and after (dark line) hydrolysis.

1996) birds and mammals (Traber and Sies 1996). The concentrations of vitamin E found in the different tissues of *L. vivipara* were within the range observed in birds (Crissey et al. 1998; Surai 2002; Karadas 2005) and mammals (Rosa et al. 2007; Il'ina et al. 2008). Our data suggest that *L. vivipara* could differ from birds and mammals in how vitamin E is allocated among different reserve tissues. Birds preferentially store vitamin E in the liver and mammals in fat stores (Rock et al. 1996; Surai 2002). In contrast, we found no significant differences in vitamin E concentrations between these two organs. Although concentrations in tail fat reserves are unknown, our results suggest that in *L. vivipara* males both liver and fat bodies function as the main vitamin E storage organs. However, more studies are needed to generalize this finding to other lizard species.

Vitamin E concentrations in *L. vivipara* did not resemble those reported in other insectivorous or related species. Namely, vitamin E concentrations in *L. vivipara* males were higher than in other insectivorous and herbivorous lizards (Cosgrove et al. 2002; Raila et al. 2002) and carnivorous, herbivorous, and omnivorous turtles (Ghebremeskel et al. 1991; Deem et al. 2006; Chaffin et al. 2008). They were, however, in the range reported for free-ranging alligators (Lance et al. 2001). Therefore, available data do not seem to support for a phylogenetic or dietary origin of vitamin E concentrations in *L. vivipara*, although only studies specifically testing these hypotheses will allow understanding of the causes. High vitamin E concentration could be also a consequence of certain environmental conditions. For example, to maintain cellular membrane fluidity in cold-water environments, some fish present high levels of unsaturated fatty acids, which require exceptionally high levels of vitamin E to avoid oxidation (Fujisawa et al. 2010). To endure low habitat temperatures and long overwintering periods, *L. vivipara* also develops highly efficient antioxidant defenses (Voituron et al. 2006). Thus, the particularly high concentrations of vitamin E

found in the tissues of *L. vivipara* could be an adaptive response allowing lizards to occupy cold habitats across Eurasia. Alternatively, high vitamin E concentrations could be the consequence of feeding lizards with wax worms, which are rich in vitamin E (Barker et al. 1998; Surai et al. 1998). However, this alternative seems unlikely because in a different study where we fed *L. vivipara* with wax worms during 40 d, plasma α-tocopherol concentration decreased over time (estimated change ± SE; –9.86 ± 2.1 μg/mL, $F_{1,41}$ = 21.95, $P < 0.001$), whereas plasma γ-(β-) tocopherol concentration slightly increased (estimated change ± SE; 0.41 ± 0.1 μg/mL, $F_{1,39}$ = 9.43, $P = 0.004$; L. M. San-Jose, personal observation). These results show that wax worms cannot explain high α-tocopherol concentrations, suggesting that wax worms may be below the nutritional α-tocopherol requirements of *L. vivipara*.

Vitamin A

Vitamin A_2 was the main vitamin A compound in the tissues of *L. vivipara* and low concentrations of vitamin A_1 were detected in the liver. Vitamin A_2 predominace has been reported in some freshwater teleosts, amphibians, and crustaceans (Wald 1939; Tsin et al. 1984; Suzuki and Eguchi 1985). In reptiles, vitamin A_1 usually predominates over vitamin A_2 (Plack and Kon 1961; Provencio et al. 1992), which is absent in most species (Thompson et al. 1999a; Thompson et al. 1999b; Thompson et al. 1999c; Raila et al. 2002). Only two studies show vitamin A_2 predominance and both studies exclusively focus on the retina (Atlas Day Gecko, *Queden-feldtia trachyblepharus*, and Green Anole, *Anolis carolinensis*; Provencio et al. 1992; Röll 2000). Thus, the predominance of vitamin A_2 in nonretinal tissues contrasts with findings reported in reptiles or in any terrestrial vertebrate.

Preferential accumulation of vitamin A_2 may occur in species feeding on vitamin A_2-rich species (Käkelä et al. 1997) or through its synthesis from provitamin A carotenoids (Gross and Budowski 1966). Because insects and other small invertebrates commonly ingested by *L. vivipara* are poor vitamin A (both A_1 and A_2) sources (Barker et al. 1998; Finke 2002), it is unlikely that vitamin A_2 predominance arises from direct vitamin A_2 intake and *L. vivipara* may therefore obtain vitamin A_2 through carotenoid metabolism (Dierenfeld et al. 2002). Among the carotenoids present in *L. vivipara* only β-carotene can be efficiently metabolize into A_2 (Morton and Creed 1939; Simpson 1983). Lutein and zeaxanthin are inefficient precursors of vitamin A, although lutein previously dehydrated to anhydrolutein can be metabolized into vitamin A_2 (Budowski and Gross 1965; Goswami and Barua 1981). However, the absence of anhydrolutein in the tissues of *L. vivipara* may discard this metabolic route. To synthesize vitamin A_2, β-carotene is first metabolized into vitamin A_1, which is finally dehydrogenated into vitamin A_2 (Gross and Budowski 1966). This metabolic route would explain why we found some small traces of vitamin A_1, although it does not explain why vitamin A_2 predominated in our study. Therefore, our data do not support that vitamin A_2 prevails in the tissues of *L. vivipara* because of dietary constrains (*i.e.*,

direct intake or increase availability of direct vitamin A_2 precursors), indicating that *L. vivipara* may preferential synthesize it, as observed in fish (Gross and Budowski 1966). The implications of increased vitamin A_2 accumulation are not clear. In species that preferentially use vitamin A_1, vitamin A_2 has been suggested to inefficiently replace health-enhancing functions of vitamin A_1, since A_2 is 49 to 60% less active in inducing and promoting growth than vitamin A_1 (Lovern et al. 1939; Shantz and Brinkman 1950). However, in an evolutionary context we would expect some beneficial effect of vitamin A_2 that counteracts its lower activity and explains why some species preferentially synthesized and accumulate it. More studies are clearly needed to completely understand the physiological and evolutionary implications of vitamin A_2 and, as shown here, *L. vivipara* can be an ideal model animal to investigate them.

Carotenoids

Similar to what has been reported in birds, amphibians, fish, and other reptiles (Czeczuga 1980; Costantini et al. 2005; McGraw 2006; Steffen et al. 2010), *L. vivipara* males seem to selectively retain more xanthophylls (lutein and zeaxanthin) than carotenes (β-carotene). This skewed carotenoid profile could result directly from the diet, given that insectivorous diets are usually richer in lutein and zeaxanthin than in β-carotene (Partali et al. 1987; Isaksson and Andersson 2007, but see Eeva et al. 2010). Alternatively, the relatively low content of β-carotene could result from a preferential use for vitamin A synthesis, which could limit its presence (Chen and Huang 2011). Xanthophyl predominance resulting from feeding lizards with wax worms (richer in lutein and zeaxanthin than in β-carotene; Barker et al. 1998; Surai et al. 1998) can be discarded since in a previous study, no differences in the carotenoid profile and in the relative carotenoid content before and after feeding lizards with wax worms for 2 mo were found (L.M. San-Jose, personal observation).

In addition to the carotenoid species detected in the current study, previous studies reported the occurrence of astaxanthin, canthaxanthin, β-cryptoxanthin, and diatoxanthin in other populations of *L. vivipara* (Czeczuga 1980; Fitze et al. 2009). These different results may reflect interpopulation variation in the available carotenoid species, but differences in analytical methodology or in peak interpretation (*e.g.*, interpretation of carotenoid ester forms as free forms; Ladislav et al. 2005) cannot be discarded. Interestingly, interpopulation differences in available carotenoid species have rarely been investigated, and to our knowledge only one study found significant differences among populations due to differences in prey diversity (Negro et al. 2000; but see Partali et al. 1987). In *L. vivipara*, prey diversity varies among populations (Avery 1966; Pilorge 1982; Heulin 1986), indicating that differences among studies in detected carotenoid species may indeed reflect interpopulation variation. Given that different carotenoid species show different biological properties (mostly in provitamin A activity and antioxidant capacity; Simpson 1983; Olson 1993; Chew and Park 2004), important implications for health are expected from interpopulation variation in carotenoid species

and *L. vivipara* can be an ideal subject to investigate them.

The observed carotenoid concentrations in plasma were within the range observed in other free-ranging lizards (Costantini et al. 2005) and those in ornamental skin were similar to reported values in ornamental traits of other lizards (Steffen and McGraw 2007) and birds (both, colored plumage and bare parts; Stradi et al. 1995; Saks et al. 2003; Butler et al. 2011). However, relative carotenoid concentration of ornamental skin compared to reserve tissues importantly differed from birds. In *L. vivipara*, ornamental skin showed similar and lower carotenoid concentrations than fat bodies and liver, respectively, whereas birds commonly accumulate higher concentrations of carotenoids in ornamental traits compared with liver and fat stores (Isaksson and Andersson 2008; McGraw and Toomey 2010). Therefore, in contrast to birds, *L. vivipara* can invest a lot of carotenoids into coloration and simultaneously maintain relatively large reserves of carotenoids. This suggests that the allocation of carotenoids to ornamentation may be less limited than in birds and that it could explain why carotenoid supplementation does not affect coloration of *L. vivipara* (Fitze et al. 2009) and other lizards (Olsson et al. 2008; Steffen et al. 2010) while it has strong effects in birds (Fitze et al. 2003; Tschirren et al. 2003).

In conclusion, we have provided information about vitamin A, vitamin E, and carotenoid content in different tissues of the common lizard, *L. vivipara*. In reptiles, especially in free-ranging squamates, little information about these micronutrients has been available and rarely have several tissues been analyzed in the same species. Our study shows that lizards deposit high concentrations of vitamin E (α- and γ-(β-) tocopherol), vitamin A (mainly A_2), and carotenoids (lutein, zeaxanthin, and β-carotene) in the liver and, less importantly, in fat bodies. In general, *L. vivipara* differs from other well-studied animals in concentration and distribution among tissues of micronutrient, suggesting that important differences in nutritional access and requirements may exist and that reptiles deserve more attention and research. Our study showed vitamin A_2 predominance in all *L. vivipara* tissues, which has been observed only in some freshwater species and in the retinas of species of two lizards. Given the carotenoids found in the tissues of *L. vivipara*, our results suggested that vitamin A_2 may be synthesized from β-carotene. This metabolic route could additionally explain why β-carotene occurs at lower concentrations than lutein and zeaxanthin and why only small amounts of vitamin A_1 were found. Together with other studies, our results indicate that important differences in carotenoid availability may exist between *L. vivipara* populations. Despite the high interest in the evolution of carotenoid-based ornaments, most studies focus on birds (Olson and Owens, 1998; Fitze and Tschirren 2006; Jacot et al. 2010). Reptiles, mainly lizards, have received attention only in the recent years (Cote et al. 2008; Olsson et al. 2008; Fitze et al. 2009; Steffen et al. 2010). Our study shows that *L. vivipara* importantly differs from birds in the allocation of carotenoids between or

namental and reserve tissues, indicating that reptiles may provide new insights in the evolution of carotenoid-based ornaments.

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Chapter IV

Dietary Lipids Reduce the Expression of Carotenoid-based Coloration in *Lacerta vivipara*

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Abstract: The importance of dietary lipids for carotenoid-based ornaments has rarely been investigated, although theory predicts that dietary lipids may control the development of these widespread animal signals. Dietary lipids have been suggested to enhance the expression of male carotenoid-based ornaments because they provide carotenoids with a hydrophobic domain that facilitates their absorption and transport. Dietary lipids may also enhance the uptake of tocopherols (vitamin E), which share common absorption and transport routes with carotenoids. Here, we test whether dietary lipids enhance carotenoid availability and male carotenoid-based colorations. We also explore the effects of dietary lipids on plasma tocopherol concentration, which allow disentangling between different pathways that may explain how dietary lipids affect ornamental expression. Following a two-factorial design, we manipulated dietary access of naturally occurring fatty acids (oleic acid) and carotenoids (lutein and zeaxanthin) and measured its effects on the circulating concentrations of carotenoids (lutein and zeaxanthin) and vitamin E (α- and γ-(β-) tocopherols) and on the ventral, carotenoid-based coloration of male common lizards (*Lacerta vivipara*). Lutein but not zeaxanthin plasma concentrations increased with carotenoid supplementation, which, however, did not affect coloration. Lipid intake negatively affected circulating concentrations of lutein and γ-(β-) tocopherol and led to significantly less orange colorations. The path analysis suggested that a relationship between the observed color change and the change in plasma concentrations of γ-(β-) tocopherol may exist. Our study shows for the first time that dietary lipids do not enhance but reduce the intensity of male carotenoid-based ornaments. Although dietary lipids affected plasma carotenoid concentration, its negative effect on coloration appeared to be linked to lower vitamin E plasma concentrations. These findings suggest that a conflict between dietary lipids and carotenoid and tocopherol uptake may arise if these nutrients are independently obtained from natural diets and that such conflict may reinforce signal honesty in carotenoid-based ornaments. They also suggest that, at least in the common lizard, sexual selection with respect to carotenoid-based coloration may select for males with high antioxidant capacity and thus for males of superior health.

Key words: Carotenoid-based ornaments; Fat intake, Protection hypothesis; Signal honesty; Sparing hypothesis; Tocopherol; Visual signals; Vitamin E.

Los Lípidos de la Dieta Reducen la Expresión de la Coloración Basada en Carotenoides de *Lacerta vivipara*

Resumen: La importancia que los lípidos de la dieta tienen sobre los ornamentos basados en carotenoides apenas ha sido investigada, aunque la teoría predice que podrían controlar el desarrollo de estas extendidas señales animales. Se ha sugerido que los lípidos de la dieta podrían mejorar la expresión de los ornamentos masculinos basados en carotenoides ya que proveen a los carotenoides con un entorno hidrofóbico que facilita su absorción y transporte. También podrían mejorar la obtención de tocoferoles (vitamina E), los cuales comparten rutas comunes de absorción y transporte con los carotenoides. Aquí testamos si los lípidos de la dieta mejoran la disponibilidad de carotenoides y los ornamentos masculinos basados en carotenoides. También exploramos los efectos de los lípidos de la dieta sobre la concentración plasmática de tocoferol, lo que permite desentrañar entre las distintas rutas que podrían explicar cómo los lípidos de la dieta afectan a la expresión ornamental. Siguiendo un diseño cruzado con dos factores, manipulamos el acceso a ácidos grasos (ácido oleico) y carotenoides (luteina y zeaxantina) que ocurren de forma natural en la dieta y medimos sus efectos sobre las concentraciones circulantes de carotenoides (luteina y zeaxantina) y de vitamina E (α- y γ-(β-) tocoferol) así como sobre la coloración ventral basada en carotenoides de los machos de la lagartija de turbera (*Lacerta vivipara*). La concentración de luteina en sangre pero no de zeaxantina aumentaron con la suplementación de carotenoides, lo que, sin embargo, no afectó a la coloración. La ingesta de lipidos afectó negativamente a las concentraciones plasmática de luteina y γ-(β-) tocoferol y resultó en coloraciones significativamente menos naranjas. El 'path analysis' sugirió que pudo existir una relación entre el cambio de color observado y el cambio en la concentración plasmática de γ-(β-) tocoferol. Nuestro estudio muestra por primera vez que los lípidos de la dieta no mejoran sino que reducen la intensidad de los ornamentos masculinos basados en carotenoides. Aunque los lípidos afectaron a la concentración de carotenoides en sangre, su efecto negativo en la coloración pareció estar ligado a una menor concentración de vitamina E. Estos hechos sugieren que un conflicto entre los lípidos de la dieta y la obtención de carotenoides y tocoferoles podría surgir si los nutrientes son obtenidos de manera independientemente en las dietas naturales y que tal conflicto podría reforzar la honestidad como señal de los ornamentos basados en carotenoides. También sugieren que, al menos en la lagartija de turbera, la selección sexual respecto a coloraciones basadas en carotenoides podría seleccionar a aquellos machos con mejor capacidad antioxidante y, por lo tanto, con mejor salud.

Introduction

Carotenoid-based ornaments displayed by birds, fish, and lizards can function as honest signals of individual quality (Olson and Owens 1998; Svensson and Wong 2011). Several (non-exclusive) mechanisms ensuring honesty have been proposed based on the assumption that carotenoid-based ornaments are costly to produce or display (Svensson and Wong 2011). Animals cannot synthesize carotenoids *de novo* and depend on dietary carotenoid intake (Goodwin 1986). The amount of carotenoids obtained with the diet may thus limit tissular carotenoid concentration (Fig. 1, path a) and thereby the development of intense carotenoid-based ornaments (Fig. 1, path b; Hill 1990; Tschirren et al. 2003). Once ingested, carotenoids can be used for ornamentation but also for other functions. Carotenoids may serve as antioxidants (Bendich 1989; Krinsky 1989; Chew and Park 2004) and, hence, individual requirements for antioxidants may determine tissular carotenoid concentration (Fig. 1, path c) and the availability of carotenoids for ornamentation (Fig. 1, path b; Lozano 1994; von Schantz et al. 1999; Pérez et al. 2008). In contrast to this hypothesis, it has been suggested that carotenoids are signalling the excellence of antioxidant resources rather than functioning as antioxidants (Hartley & Kennedy 2004). Hartley & Kennedy (2004) proposed that carotenoids may be vulnerable to oxidation and argued that "given that oxidation of carotenoids alters or destroys their colour (*i.e.*, bleaches them), preservation of their colour intensity would indicate the possession of efficient means for their protection". In this scenario, colour intensity would be an indicator of antioxidant capacity but carotenoids themselves would not be responsible for antioxidation (Hartley and Kennedy 2004).

According to the above-stated hypotheses, carotenoid-based ornaments may depend on access to carotenoids as well as antioxidants, and therefore on factors modulating absorption, transport, and metabolism of carotenoids and antioxidants (Hill 2000; McGraw and Parker 2006; Fitze et al. 2007). Nutritional and biomedical literature indicates that dietary lipid intake is essential for an efficient assimilation of dietary carotenoids (Erdman et al. 1993; Parker 1996; van het Hof et al. 2000; Yonekura and Nagao 2007). Lipids provide carotenoids with a hydrophobic domain that favors their emulsion from the intestinal lumen, their inclusion into mixed micelles, and their posterior absorption in the duodenal cells. After absorption, dietary lipids enhance carotenoid transport by stimulating the formation of carotenoid carriers; chylomicrons and lipoproteins (Borel et al. 1998; Silva et al. 2003). Consequently, increased lipid intake may lead to higher tissular carotenoid availability, as demonstrated in humans and laboratory mammal species (Fig. 1, path d; Dimitrov et al. 1988; Brown et al. 2004; Unlu et al. 2005). The importance of dietary lipids has been rarely investigated in species with carotenoid-based ornaments, which may importantly differ from mammals in carotenoid dynamics (Hill 1999). The enhancing effect of dietary lipids on carotenoid assimilation and transport may translate into improved ornamental expresion. Moreover, its effect may be higher at low than at high dietary carotenoid

concentrations (Ribaya-Mercado 2002), suggesting that the scarcer carotenoids are in the diet the stronger dietary lipids may affect carotenoid-based ornaments. Thus, between-individual variation in dietary lipid access may help to understand how differences in carotenoid availability and carotenoid-based ornaments arise and how these differences become related to differences in individuals' quality.

Because tocopherols (*i.e.*, vitamin E) share absorption and transport routes with carotenoids, dietary lipids may also increase tocopherol uptake (Fig. 1, path e; Rock et al. 1996). Tocopherols are potent lipid-soluble antioxidants of dietary origin that may preferentially bolster antioxidant defences (Rock et al. 1996). In some species, enhanced tocopherol levels may therefore lead to the sparing of carotenoids, which can then be used for pigmentation (Fig. 1, path f-c-b; Pérez-Rodríguez 2009). Alternatively, enhanced tocopherol levels may increase the protection of carotenoids from oxidation, and thereby affect carotenoid-based ornaments (Fig. 1, path g-b; Hartley and Kennedy 2004).

On the other hand, if dietary lipids lead to an increase in carotenoid concentration, they may also indirectly decrease tissular tocopherol concentrations. Thus, if carotenoids require antioxidant protection, enhanced carotenoid levels may increase the consumption of tocopherols due to the toxic workings of carotenoid oxidation (Fig 1, path d-g; Palozza 1998). Alternatively, if carotenoids function as antioxidants, enhanced carotenoid concentrations due to increased lipid uptake may spare other antioxidants from antioxidant funtions,

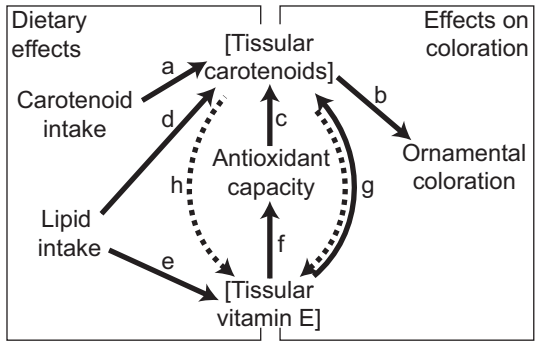

FIG. 1.—Hypothesized pathways explaining how dietary lipids and carotenoids may affect tissular carotenoid and vitamin E concentrations and carotenoid-based ornaments (the figure only includes pathways important for this study). Dietary effects on tissular concentration of carotenoids and vitamin E are shown in the left panel, effects of tissular carotenoid and vitamin E use is shown in the middle, and effects on coloration are shown in the right panel. Continuous lines indicate positive and dotted lines negative relationships. Carotenoid intake enhances tissular carotenoid concentrations (path a). Lipid intake determines carotenoid and tocopherol absorption and transport and thereby tissular carotenoid or tocopherol concentration (paths d and e). Antioxidant capacity may affect tissular carotenoids (path c) and vitamin E (path h) and both the sparing effect (path i) and the protection hypothesis (path d) may explain the link between tissular tocopherols and carotenoids. All pathways may finally affect ornamental carotenoid-based coloration through their effects on tissular carotenoid concentration (path b).

which is known as homeostatic sparing (Svensson and Wong 2011). According to the sparing hypothesis, non-carotenoid antioxidants, including tocopherol, are preferentially used if carotenoids are scarce, and down-regulated if carotenoids are plentiful, which is suggested to explain the negative correlations that have been previously observed between carotenoid intake and the tocopherol concen-

tration of some tissues (Fig. 1, path d-h; Surai 2002).

To our knowledge, only one study has considered the role of dietary lipids on the expression of carotenoid-based ornaments. McGraw & Parker (2006) showed that dietary cholesterol supplementation enhances blood carotenoid content and carotenoid-based coloration of *Taenopygia guttata* (zebra finch). Because *T. guttata* does not naturally ingest cholesterol (McGraw and Parker 2006), these findings need to be confirmed with naturally ingested lipids. Further, no evidence exists for the existence of similar effects in other species and thus the generallity of these findings remains unknown.

Here, we investigate the effects of dietary lipids on carotenoid-based ornaments using male common lizards, *Lacerta vivipara*, as a study organism. To investigate by which mechanisms dietary lipids may affect coloration we simultaneously supplemented lizards with lipids and carotenoids using a 2 × 3 factorial design and measured plasma carotenoid and tocopherol concentrations. Male common lizards exhibit a ventral, yellow orange coloration that is based on carotenoids (Fitze et al. 2009). We predicted that dietary lipid supplementation positively affects plasma carotenoid concentrations (Fig. 1, path d). We also expect that differences between lizards supplemented with dietary lipids and control lizards will increase with decreasing amounts of dietary carotenoids, *i.e.*, when carotenoids are scarcer. We further predict that enhanced carotenoid concentrations positively affect ornamentation, through increased carotenoid incorporation into ornamental coloration (Fig.1, path b). We also predicted that dietary lipids may directly increase plasma tocopherol concentrations (Fig. 1, path e) or indirectly (*i.e.*, through enhancing carotenoid uptake) decrease them as predicted by the protection hypothesis (Fig. 1, path d-g) or the sparing effect (Fig. 1, path d-h). If enhanced carotenoid uptake mediates indirect effects of dietary lipids in plasma tocopherol concentrations, carotenoid supplementation should also negatively affect plasma tocopherol concentration (Fig. 1, path a-h or a-g).

Materials and Methods

Species Description, Study Site, and Breeding Conditions

The common lizard is a small ground-dwelling lizard that inhabits humid habitats across Europe and Asia (Sindaco and Jeremcenko 2008). It is an insectivore species that mainly preys on spiders and homoptera (Avery 1966; Avery 1971; Heulin 1986) but also on larger preys like earthworms (L. M. San-Jose, *personal observation*). In the first year of life, males develop a yellow-orange carotenoid-based coloration that plays a role in sex recognition and female mate choice (Bauwens et al. 1987; Fitze et al. 2009; Cote et al. 2010)). Male coloration is mantained all throughout the year and does not fade after the breeding season. The presence of other yellow-orange pigments, like pteridines, has been discarded (Fitze et al. 2009).

On 2 August 2007, 42 adult males were captured in a natural population located at Puerto de Ibañeta, Spanish Pyrenees (43°1'N, 1°19'W). The experiment was conducted from

August to end of September, because food intake during these months (*i.e.*, before hibernation) determines common lizard's condition in spring and, hence, in the mating period (Avery 1970; Roig et al. 2000; Bleu et al. 2011). After capture, animals were moved to the laboratory at the Instituto Pirenaico de Ecología-CSIC (Jaca, Spain) and snout to vent length (SVL, to the nearest 1 mm) and body mass (to the nearest 1 mg) were measured. Lizards were individually housed in *terraria* (25 × 15 × 15 cm) containing two different shelters and peat soil as substrate. A 40 W bulb provided light and heat for 8 h a day and a UV light (5% UV-B and 30% UV-A; Sylvania Reptistar, London, UK) illuminated the terraria during 2 h a day. Lizards were left to acclimate to laboratory conditions for 10 days. During this period, water was provided *ad libitum* and lizards were fed with a *Galleria mellonella* (wax worm) larva every four days.

Diet Manipulation

During 31 days, dietary lipids and carotenoids were manipulated following a 2 × 3 factorial design. Half of the lizards were randomly assigned to a fat-supplemented group (N = 21) and the other half to an unsupplemented group (N = 21). Within fat-supplemented and unsupplemented groups, lizards were randomly assigned to three different carotenoid supplementation groups (N = 7 per carotenoid and fat supplementation group). Starting on day 1, lizards were fed every two days with a *G. mellonella* larvae injected with the corresponding fat and carotenoid supplementation. Fat and carotenoids were injected into *G. mellonella* larvae to assure that carotenoids could dissolve in dietary lipids. Body mass was periodically measured (days 1, 9, 22, and 31) and the number of larvae eaten was counted to investigate treatment effects on appetite.

Lizards were supplemented with oleic acid, a monounsaturated fatty acid that predominates in insectivore diets (Barlow 1963; Fast 1966; Schneider and Dorn 1994; Buckner 2004; Speake et al. 2004; Bashan 2005; Michaud and Denlinger 2006; Khani et al. 2007) and reptile reserve tissues (Ballinger et al. 1992; Cartland-Shaw et al. 1998; Thompson et al. 2001). Fat-supplemented lizards were fed with a *G. mellonella* larvae injected with 0.12 mL pure olive oil (72 % oleic acid), while unsupplemented lizards were fed with larvae injected with 0.12 mL of distilled water. Thus, lizards in the fat supplemented group received approximately three times more oleic acid and 1.5 times more fat than lizards in the unsupplemented group (Finke 2002).

Lizards were supplemented with lutein and zeaxanthin, which account for 99.1 % of the plasma carotenoids present in common lizards of Pyrenean populations (San-Jose, et al. 2012). Lizards supplemented with a high dose of carotenoids were fed with *G. mellonella* larvae injected with 30 µL of a solution containing 2 mg lutein and zeaxanthin beadlets (lutein: 5.58 % and zeaxanthin: 0.44 %, Hoffmann-La Roche Ltd., Basel, Switzerland) in 1 mL distilled water. Lizards supplemented with an intermediate dose of carotenoids were fed with *G. mellonella* larvae injected with 30 µL of a solution containing 1 mg lutein and zeaxanthin beadlets and 1 mg control beadlets diluted in 1 mL distilled water. Finally, lizards in a control-dose group were fed with *G. mel-*

lonella larvae injected with 30 µL of a solution of 2 mg control beadlets in 1 mL distilled water (Finke 2002).

HPLC Analyses

On day 1, 21, and 36, blood samples were taken from the retro-orbital sinus using heparinized microcapillaries. Blood was thereafter centrifuged (5 min at 8900 g) and the obtained plasma was stored at –80 °C. HPLC analyses were run on a subset of 21 randomly selected males. There were no statistical differences in SVL, body mass, and body condition between the subset and the rest of the animals (all $P > 0.41$).

Plasma concentrations of lutein, zeaxanthin, and α- and γ-(β-) tocopherol were analyzed using HPLC (Olmedilla et al. 1997). Plasma samples were mixed with 0.1 mL distilled water and 0.2 mL ethanol (EtOH) and extracted twice with 0.5 mL methylene chloride:hexane (1:5). Organic phases were pooled, evaporated to dryness, and reconstituted in tetrahydrofuran (THF):EtOH to be injected onto the HPLC column. The chromatographic system consisted of a Spheri-5-ODS column (Applied Biosystems, San Jose, CA, USA) with gradient elution of acetonitrile:methanol (85:15; 5 min) to acetonitrile:methylene chloride:methanol (70:20:10; 20 min). Ammonium acetate (25 mM) was added to the methanol. Carotenoid detection was carried out with a photodiode array (model 2996; Waters Associates, Milford, MA, USA) set at 450 nm. Tocopherols were detected using the same photodiode set at 295 nm and using a fluorescence detector (excitation: 290 nm, emission: 330 nm). Identification of the compounds was carried out by comparing retention times with those of authentic standards and on-line UV-visible spectra. The accuracy of the analytical method was periodically verified through our participation in the Fat-Soluble Quality Assurance Program (National Institute of Standards and Technology, Gaithersburg, MD, USA).

Color Measurements

Coloration was measured using a tristimulus scoring system based on measures of hue, chroma, and brightness (Endler 1990). Coloration was measured on day 1, 10, 22, and 31 using an USB4000 spectrometer (Ocean Optics Inc., Dunedin, FL, USA) attached to a deuterium tungsten halogen light source (DT-MINI-2-GS, Ocean Optics Inc.) by an optic fibre probe (QR400-7-UV/VIS-BX, Ocean Optics Inc.). Spectral reflectance of the throat scales, anal plate, and inner ventral scales was measured in relation to a diffuse white standard (WS-1, Ocean Optics Inc.) using an angle of 45 °. All measurements showed high repeatability (repeatability based on two repeated measurements: $F_{10,11} \geq 5.23$, $P \leq 0.01$, $r \geq 0.68$; Lessells and Boag 1987).

Statistics

At the beginning of the experiment, there were no significant differences between treatment groups in SVL, body mass, and body condition (all $P > 0.57$). Two lizards died during the experiment and were excluded from all analyses. Treatment effects on appetite were analyzed using ANOVAS with fat and carotenoid treatment and their interaction as factors. Treatment effects on repeated measures of body mass, plasma concentrations of lutein,

zeaxanthin, α- and γ-(β-) tocopherol, and color variables were analyzed using the MIXED procedure implemented in SAS 9 system (SAS Institute Inc., Cary, NC, USA; Littell et al. 1998). Full models included fat and carotenoid treatment and their interaction as fixed factors and individual as random effect. Time effects were investigated by fitting linear and quadratic trends and their interaction with fat and carotenoid treatment. The covariance structure of each model was selected following the Akaike criterion (Wolfinger 1996; Littell et al. 1998). Model simplification was carried out using backward elimination of non-significant terms. To elucidate by which mechanisms fat and carotenoid-supplementation affected coloration, we ran path analyses using multivariate regression models based on standardized variables (Fitze and Le Galliard 2008). Path analysis was used to distinguish between direct and indirect treatment effects on coloration, *e.g.*, treatment effects on coloration owing to treatment effects on tissular carotenoid and/or tocopherol concentrations. Moreover, they unravel the relative importance of the applied treatments and of the changes (final – initial measures) in tocopherol and carotenoid concentrations that result in coloration change.

The significance level was set at $P = 0.05$ (two-tailed) and it was adjusted following Hochberg procedures (Hochberg 1988) to control family-wise Type I error rate when using multiple contrasts. If necessary, variables were transformed to meet model assumptions. Some models did not meet the homocedasticity assumption and weighted least-square regressions were fitted instead (Neter et al. 1996). Cohen's *d* estimates of effect sizes and 95 % confidence intervals were obtained following calculations for mixed models described in Nakagawa & Cuthill (2007).

Results

Effects on Appetite and Body Mass

Over the course of the experiment, lizards ate a total of 12 ± 0.5 (mean ± SE) larvae. Appetite was not significantly affected by fat treatment ($F_{1,36} = 0.21$, $P = 0.65$), carotenoid treatment ($F_{2,36} = 1.24$, $P = 0.30$), and their interaction ($F_{2,34} = 1.10$, $P = 0.35$). During the first weeks of the experiment, lizards showed a significant decrease in body mass, that was followed by a slight increase at the end of the experiment (time: estimate ± SE = –0.177 ± 0.062 g, $F_{1,110} = 7.26$, $P = 0.008$; time^2: estimate ± SE = 0.039 ± 0.011 g, $F_{1,110} = 43.33$, $P < 0.001$). Body mass was not significantly affected by fat treatment (fat treatment × time: $F_{1,110} = 0.78$, $P = 0.38$; fat treatment × time^2: $F_{1,110} = 2.90$, $P = 0.09$), carotenoid treatment (carotenoid treatment × time: $F_{2,110} = 1.51$, $P = 0.22$; carotenoid treatment × time^2: $F_{2,110} = 0.18$, $P = 0.83$), or the three-way interactions between fat treatment, carotenoid treatment, and time or time^2 (all $P > 0.10$).

Effects on Plasma Carotenoids and Tocopherols

Over the course of the experiment, fat and carotenoid treatment significantly affected plasma lutein concentration in a quadratic manner (Table 1; Fig. 2) and the interaction between the two treatments was not significant (all $P > 0.75$). Fat-supplemented lizards showed a significant decrease in plasma lutein concentration over the course of the experi-

ment ($F_{1,19}$ = 9.52, P = 0.006; Fig. 2a). In contrast, plasma lutein concentrations of unsupplemented lizards increased until day 21, when they reached a maximum and started to decrease (time: $F_{1,20}$ = 10.74, P = 0.004; time^2: $F_{1,20}$ = 9.71, P = 0.006). Plasma lutein concentrations of lizards supplemented with high and intermediate doses of carotenoids increased until day 21 and then decreased (high dose; time: $F_{1,10}$ = 13.10, P = 0.005; time^2: $F_{1,10}$ = 12.87, P = 0.005, intermediate dose; time: $F_{1,14}$ = 6.11, P = 0.027; time^2: $F_{1,14}$ = 6.45, P = 0.023; Fig. 2b). In the control-dose group, lutein concentration decreased from days 1 to 21 and remained low until day 36 (time: $F_{1,13}$ = 6.26, P = 0.026). Individual contrasts showed that the changes observed in the high and intermediate dose groups were significantly different from those observed in the control-dose group (high dose *vs*. control dose; time: $F_{1,34}$ = 29.38, P < 0.001; time^2: $F_{1,34}$ = 25.20, P < 0.001, intermediate dose *vs*. control dose; time: $F_{1,34}$ = 16.81, P < 0.001; time^2: $F_{1,34}$ = 14.14, P <

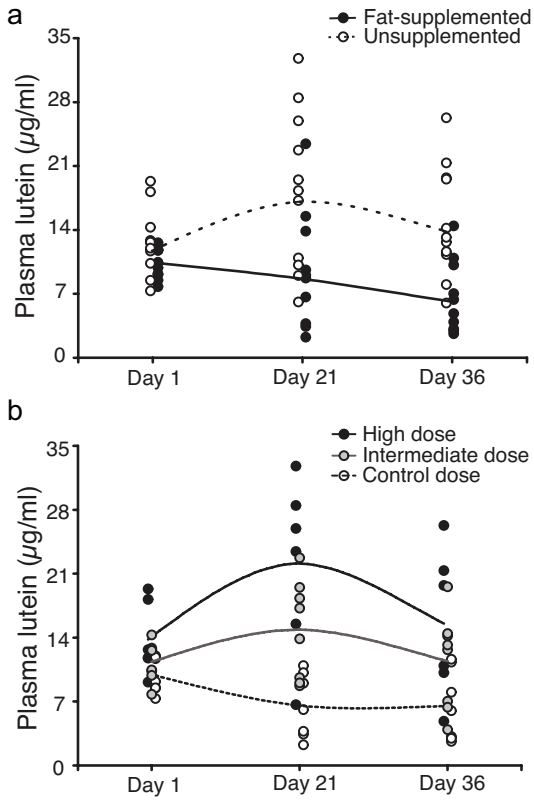

FIG. 2.— Effects of fat (a) and carotenoid (b) treatment on plasma lutein concentration. Points represent observed data and lines represent model predictions for each treatment level.

TABLE 1.—Treatment effects on plasma concentration of lutein and zeaxanthin. Test statistics of mixed models with linear and quadratic time effects are given. Bold values are statistically significant. Effect size (*d*) and 95 % confidence intervals are given for statistically significant parameters.

	Lutein				Zeaxanthin			
Parameter	Test statistic	*P*	Effect size (*d*)	95 % CI	Test statistic	*P*	Effect size (*d*)	95 % CI
Time	$F_{1,34}$ = 6.86	**0.013**	0.56	0.11-1.07	$F_{1,40}$ = 4.62	**0.037**	0.41	0.02-0.84
Time^2	$F_{1,34}$ = 8.55	**0.006**	0.62	0.17-1.16	$F_{1,40}$ = 2.95	0.093		
Fat treatment × time	$F_{1,34}$ = 9.71	**0.004**	0.65	0.21-1.08	$F_{1,37}$ = 1.67	0.204		
Fat treatment × time^2	$F_{1,34}$ = 6.15	**0.018**	0.51	0.09-0.94	$F_{1,34}$ = 0.40	0.532		
Carotenoid treatment × time	$F_{2,34}$ = 17.33	<**0.001**	1.67[a]	0.94-2.38	$F_{2,37}$ = 1.87	0.168		
			1.11[b]	0.51-1.70				
Carotenoid treatment × time^2	$F_{2,34}$ = 15.02	<**0.001**	1.54[a]	0.83-2.24	$F_{2,35}$ = 0.94	0.402		
			1.02[b]	0.43-1.60				

[a] Estimate for high carotenoid dose group *vs.* control dose group.

[b] Estimate for intermediate carotenoid dose group *vs.* control dose group.

0.001), but not between the carotenoid-supplemented groups (high dose *vs*. intermediate dose; time: $F_{1,34}$ = 2.72, P = 0.11; time^2: $F_{1,34}$ = 2.43, P = 0.13). Zeaxanthin concentration in plasma significantly decreased over the course of the experiment and it was not affected by any of the treatments (Table 1) or their interaction (all P > 0.34).

Fat treatment significantly affected plasma γ-(β-) tocopherol concentration (time: $F_{1,39}$ = 9.43, P = 0.004, effect size d = 0.24, 95 % CI = 0.07-0.40; time^2: $F_{1,39}$ = 5.30, P = 0.027, effect size d = 0.18, 95 % CI = 0.02-0.33; fat treatment × time: $F_{1,39}$ = 13.60, P < 0.001, effect size d = 0.28, 95 % CI = 0.12-0.45; Fig. 3). Plasma γ-(β-) tocopherol concentration significantly increased in unsupplemented lizards, while in fat supplemented lizards it increased less until day 21 and slightly decreased thereafter. Carotenoid treatment did not significantly affected γ-(β-) tocopherol concentration (carotenoid treatment × time: $F_{2,36}$ = 0.58, P = 0.57) and the interaction between fat and carotenoid treatment was not significant (all P > 0.43). α-Tocopherol concentration decreased during the experiment (time estimate ± SE = –9.856 ± 2.104 μg/mL; $F_{1,41}$ = 21.95, P < 0.001) and was not significantly affected by fat treatment (fat treatment × time: $F_{1,35}$ = 0.02, P = 0.89), carotenoid treatment (carotenoid treatment × time: $F_{2,38}$ = 0.55, P = 0.58), or their interaction (all P > 0.27).

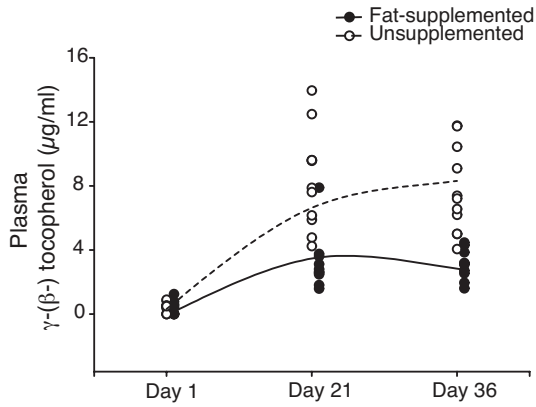

FIG. 3.— Effects of fat treatment on plasma γ-(β-) tocopherol concentration. Points represent observed data and lines represent model predictions for each treatment level.

Effects on Coloration

Fat treatment significantly affected anal plate and throat scale hue (Table 2). Over the course of the experiment, hue of both anal plate and throat scale decreased more (*i.e.*, became more orange) in unsupplemented than in fat-supplemented lizards (Fig. 4). A similar but not significant tendency was also found for ventral scale hue (Table 2; Fig. 4). Carotenoid treatment and its interaction with fat treatment did not significantly affect coloration (all $P \geq 0.63$) and there were no significant treatment effects on chroma (all $P \geq 0.13$) and brightness (all $P \geq 0.33$) in any of the measured body parts.

Path analysis (Fig. 5, Table 3) revealed that fat treatment negatively affected the change in plasma lutein concentration, *e.g.*, lutein concentration decreased in fat-supplemented lizards and increased in unsupplemented lizards (see also Fig. 2). Similarly, fat treatment negatively affected the change in blood γ-(β-) tocopherol concentration, *e.g.*, tocopherol concentration increased less in fat-supplemented lizards than in unsupplemented lizards (see also Fig. 3). Fat treatment did not significantly affect the change in zeaxanthin

TABLE 2.— Effects of fat and carotenoid supplementation on common lizard coloration. Test statistics of mixed models with linear and quadratic time effects are shown for hue measured on the anal plate, throat scales, and ventral scales. Bold values are statistically significant. Effect size (*d*) and 95 % confidence intervals are given for statistically significant parameters.

	Anal plate				Throat scales				Ventral scales			
Parameter	Test statistic	*P*	Effect size (*d*)	95 % CI	Test statistic	*P*	Effect size (*d*)	95 % CI	Test statistic	*P*	Effect size (*d*)	95% CI
Time	$F_{1,117} = 33.21$	**< 0.001**	0.88	0.53-1.31	$F_{1,117} = 26.7$	**< 0.001**	0.81	0.46-1.22	$F_{1,117} = 44.03$	**< 0.001**	0.97	0.62-1.40
Time^2	$F_{1,117} = 17.95$	**< 0.001**	0.63	0.31-0.98	$F_{1,117} = 12.52$	**< 0.001**	0.53	0.22-0.87	$F_{1,117} = 24.27$	**< 0.001**	0.69	0.39-1.03
Fat treatment × time	$F_{1,117} = 6.55$	**0.012**	0.37	0.08-0.65	$F_{1,117} = 7.36$	**0.008**	0.40	0.11-0.69	$F_{1,117} = 3.00$	0.086		
Fat treatment × time^2	$F_{1,116} = 0.24$	0.623			$F_{1,116} = 0.78$	0.379			$F_{1,116} = 0.22$	0.641		
Carotenoid treatment × time	$F_{2,114} = 0.26$	0.771			$F_{2,114} = 0.47$	0.629			$F_{2,114} < 0.001$	0.999		
Carotenoid treatment × time^2	$F_{2,112} = 0.26$	0.774			$F_{2,112} = 0.25$	0.776			$F_{2,112} = 0.01$	0.994		

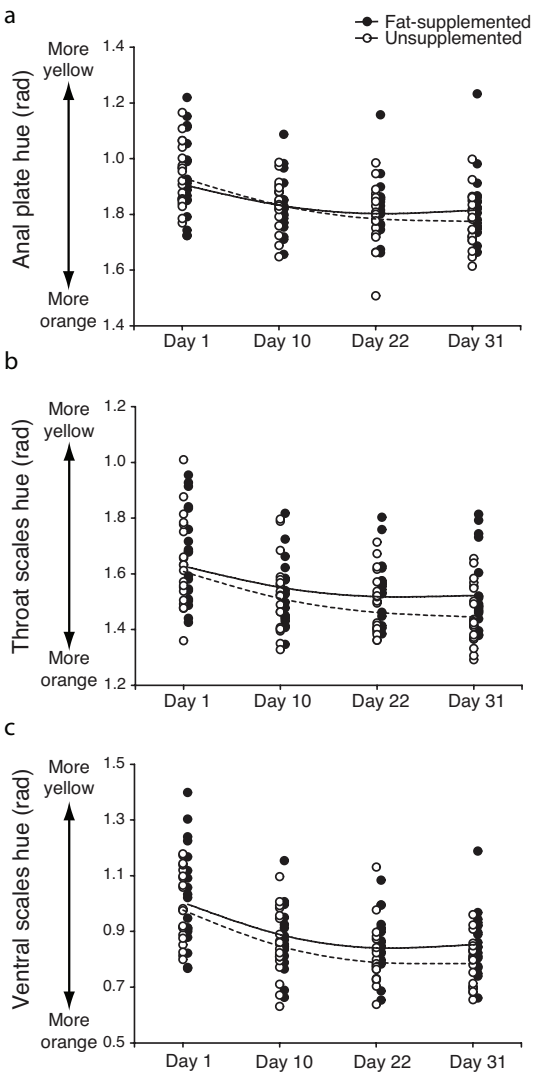

FIG. 4.—Effects of fat treatment on anal plate (a), throat scale (b), and ventral scale (c) hue. Points represent observed data and lines represent group specific model predictions.

and α-tocopherol concentrations (Table 3). Carotenoid treatment positively affected the change in plasma lutein concentration and negatively affected plasma zeaxanthin concentration (Fig. 5). Individual contrasts showed an increase in lutein concentration in the high dose group compared to lizards supplemented with intermediate (estimate ± SE = 3.98 ± 1.60 μg /mL, t_{16} = 2.49, P = 0.048) and control doses (estimate ± SE = 6.05 ± 1.53 μg/mL, t_{16} = 3.95, P = 0.003). Lutein change was not significantly different between lizards fed with intermediate and control doses (t_{16} = 1.39, P = 0.18). The high dose group showed a higher decreased in plasma zeaxanthin than lizards in the intermediated dose group (estimate ± SE = –0.76 ± 0.28 μg/mL, t_{17} = 2.72, P = 0.044) and tended to show a higher decrease than lizards in the control dose group (estimate ± SE = –0.65 ± 0.31 μg/mL, t_{17} = 2.08, P = 0.08). Carotenoid treatment did not significantly affected the change in plasma α- and γ-(β-) tocopherol concentrations. The change in lutein concentration significantly explained changes in zeaxanthin concentrations and *vice versa*. Changes between tocopherols and between tocopherols and carotenoids were not significantly related.

The change in plasma lutein, α-tocopherol, and zeaxanthin concentrations did not explain the change in hue on any of the three body parts. The change in γ-(β-) tocopherol was negatively related to the hue change on ventral and throat scales and a trend existed on the anal plate. The smaller increase in γ-(β-) tocopherol concentration observed in the fat-supplemented group let to a smaller decrease in hue and thus to less orange coloration (Fig. 6). In contrast, individuals of the control-supplemented group showed a bigger increase in γ-(β-) tocopherol concentration that let to a bigger decrease in hue and thus to more orange coloration (Fig. 6). No direct effect of the fat treatment existed on coloration (Table 3; Fig. 5).

TABLE 3.—Results from path analysis discerning between direct treatment effects on changes in hue of the anal plate, throat scales, and ventral scales and indirect treatment effects mediated by changes in carotenoid and tocopherol concentrations. Bold values are statistically significant.

	Independent variable					
	Direct effects		Indirect effects			
Dependent variables	Fat treatment	Carotenoid treatment	ΔLutein	ΔZeaxanthin	Δγ-(β-) Tocopherol	Δα-Tocopherol
ΔLutein	$F_{1,16}$ = **20.78*****	$F_{2,16}$ = **8.36****	—	$F_{1,16}$ = **9. 58****	$F_{1,15}$ = 1.56	$F_{1,14}$ = 1.06
ΔZeaxanthin	$F_{1,16}$ = 2.04	$F_{2,17}$ = **3.93***	$F_{1,17}$ = **14.63*****	—	$F_{1,15}$ = 1.41	$F_{1,14}$ = 0.06
Δγ-(β-) Tocopherol	$F_{1,19}$ = **23.56*****	$F_{2,17}$ = 1.19	$F_{1,16}$ = 1.81	$F_{1,15}$ = 1.41	—	$F_{1,14}$ = 0.58
Δα-Tocopherol	$F_{1,16}$ = 0.62	$F_{2,18}$ = 0.65	$F_{1,15}$ = 0.02	$F_{1,14}$ = 0.05	$F_{1,17}$ = 0.45	—
ΔAnal plate hue	$F_{1,15}$ = 0.50	$F_{2,16}$ = 1.72	$F_{1,16}$ = 0.83	$F_{1,16}$ = 0.14	$F_{1,16}$ = 4.02$^{m.s.}$	$F_{1,16}$ < 0.01
ΔThroat scales hue	$F_{1,18}$ = 1.41	$F_{2,16}$ = 2.33	$F_{1,16}$ = 2.81	$F_{1,16}$ = 0.19	$F_{1,16}$ = 5.67*	$F_{1,16}$ = 0.76
ΔVentral scales hue	$F_{1,15}$ = 0.88	$F_{2,17}$ = 1.89	$F_{1,16}$ = 1.72	$F_{1,16}$ = 0.02	$F_{1,16}$ = 9.66**	$F_{1,16}$ < 0.01

* P<0.05, ** P<0.01, *** P <0.001, m.s. marginally significant 0.05 < P ≤ 0.06

DISCUSSION

Here, we show that increased lipid intake reduced plasma concentration of lutein, which is the predominant carotenoid of common lizard tissues (San-Jose et al., 2012). The absence of a significant interaction between fat and carotenoid treatment suggests that lipid effects on plasma carotenoid content may be independent of carotenoid intake and, hence, that lipid effects may not depend on enviromental scarcity of carotenoids or individual differences in obtaining carotenoids. Decreased plasma lutein concentration owing to lipid intake may have resulted from lipid effects on lutein absorption, transportation, or excretion (Parker 1996). If dietary lipids impaired carotenoid absorption or increased carotenoid excretion, we would have expected no differences between fat treatment groups in the carotenoid control group, given that the carotenoid control group was provided with a neglectable quantity of carotenoids (0.53 % to 0.86 % of the carotenoid reserves of common lizards; San-Jose et al., 2012). On the contrary, if lipids impaired post-absorption mechanisms (*e.g.*, carotenoid transport), we expected a similar effect of fat treatment in all carotenoid treatment groups. The absence of a significant interaction between lipid and carotenoid treatment therefore suggests that dietary lipids may have affected lutein during post-absorption but not during absorption or excretion. Because lipids and carotenoids are transported by plasma lipoproteins, which are formed after absorption, increased competition between lipids and carotenoids for inclusion into lipoproteins may have compromised carotenoid transport capacity in fat supplemented lizards (Parker 1996). Our findings are not in line with previous studies conducted in humans and laboratory mammal species, where dietary lipids did enhance ca-

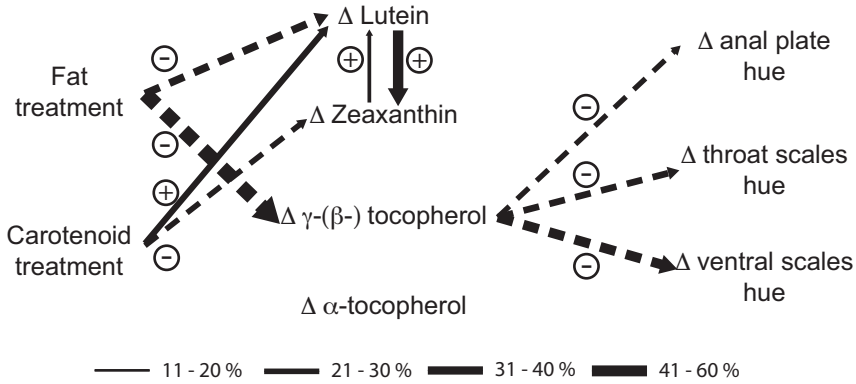

FIG. 5.—Path diagram showing significant direct and indirect treatment effects on anal plate, throat scale, and ventral scale hues. Arrows indicate significant and marginally significant effects. Negative and positive effects are indicated using dashed and continuous arrows, respectively. The direction of treatment effects is given for supplemented groups relative to the unsupplemented group (*e.g.* supplemented group > unsupplemented group is referred to as a positive effect). For the zeaxanthin change, the direction of the carotenoid treatment is given for the high carotenoid dose group relative to the other groups. Arrow width is proportional to the explained variance (scale provided in the figure).

rotenoid uptake (Yonekura and Nagao 2007). In contrast to mammals, species displaying carotenoid-based ornaments usually present higher circulating carotenoid concentrations, which has been suggested to result from important differences in carotenoid dynamics and utilization (review in Hill 1999). Our findings may also reflect such differences, supporting that extrapolation from observations made in mammals to species with carotenoid-based ornaments should be cautiously carried out (Hill 1999). Additional studies comparing the effect of dietary lipids in diferent taxa as well as in different tissues are therefore needed. Additionally, the negative effects of dietary lipids may be ultimately modulated by hormones like testosterone, which regulates lipoprotein status and which potential effects should be also addressed in future studies (McGraw et al. 2006).

Fat treatment-induced tocopherol changes paralleled carotenoid changes, which is in line with the idea of shared absorption and transportation routes (Borel 2003). In plasma, we found no evidences for homeostatic sparing or the protection hypothesis, although homeostatic sparing may exist among other, here not analysed, tissues or antioxidant compounds (Fig. 1, paths h and g; Svensson and Wong 2011). Carotenoid supplementation did not induce changes in plasma tocopherol concentration, suggesting that carotenoids did not substitute tocopherols as antioxidants or that tocopherols were not consumed by toxic workings of carotenoid oxidation (Palozza 1998). As suggested for carotenoids, negative interactions between lipids and tocopherols during absorption, excretion, or transport are more likely to explain the negative relationship between lipid ingestion and γ-(β-) tocopherol plasma concentration.

As previously observed in common lizards,

carotenoid supplementation increased lutein plasma concentration while supplementation with no carotenoids decreased lutein plasma concentration (Fitze et al. 2009). Our study is therefore in line with studies on birds and fish showing that circulating carotenoid levels reflect the amount of dietary carotenoids (Fig 1, path a; Hill et al. 1994; Grether et al. 1999; Hill et al. 2002; Clotfelter et al. 2007). Despite experimental zeaxanthin supplemention, no direct supplementation effect on plasma zeaxanthin concentration was found. Similarly, dietary lipids did also not affect plasma zeaxanthin concentration while significantly affected lutein concentration. These findings suggest that plasma zeaxanthin levels may be less flexible than plasma lutein levels. In line with these findings, the path analysis suggested that carotenoid supplementation may have negatively affected zeaxanthin plasma concentration, suggesting that lutein may be favoured over zeaxanthin in potential competitive interactions during absorption or transport (Erdman et al. 1993).

Although decreased carotenoid availability directly explains color fading in many species showing carotenoid-based ornaments (*e.g.*, Tschirren et al. 2003), it was not responsible for the color changes mediated here by lipid intake. The determination of carotenoid-based ornaments has been suggested to be conserved in different vertebrates groups, like birds, fish, and reptiles (Peters 2007). However, our study shows that manipulation of carotenoid availability does not affect the expression of carotenoid-based ornaments, which is in sharp contrast to findings in birds and fish (Grether et al. 2001; McGraw et al. 2002; McGraw et al. 2004). This result is congruent to previous experimental studies conducted in the common lizard (Fitze et al. 2009) and with studies on other lizard species, where carotenoid administration did also not affected coloration (Olsson et al. 2008; Steffen et al. 2010). Therefore, to date, there is no evidence that carotenoid availability limits the expression of carotenoid-based coloration in lizards (Fig. 1, path b) and, hence, the hypothesis that coloration reflects individual differences in carotenoid ingestion or in carotenoid allocation may be not supported in lizards. To fully test this hypothesis in lizards, it is still necessary to address the importance of carotenoid availability during maturation and, hence, when most lizard species develop bright colorations for the first time.

Enhanced lipid intake could have directly affected coloration through increasing the amount of lipids deposited in the epidermis (Lillywhite 2006) and/or the flexibility of the chromatophore cell membranes (Geiser 1991). However, a direct effect of lipid intake on coloration was not supported by the path analysis. The path analysis indeed showed that the negative effect of fat treatment on ventral coloration was related with the change observed in plasma γ-(β-) tocopherol concentration, suggesting that lipid intake negatively affected tocopherol concentrations and thereby led to less orange and, hence, less sexually attractive coloration (Fitze et al. 2009). Our findings are in line with previous findings in the common lizard. Cote et al. (2010) showed that common lizards with less orange colorations had higher concentrations of lipid peroxidation. Increased lipid peroxida-

tion usually diminishes circulating concentrations of tocopherols (Ibrahim et al. 1997), given that tocopherols specifically protect lipids from free radical attack (Rock et al. 1996). Consequently, in our study and Cote's et al. (2010), tocopherol availability may explain the observed results. Our findings also resemble those found in other lizard species. For instance, a relationship between tocopherol concentrations and coloration was observed in male *Lacerta lepida* (ocellated lizard; Martín and López 2010), and between antioxidant capacity and coloration in *Ctenophorus pictus* (Australian painted dragon lizard) whose carotenoid-based coloration also did not depend on dietary carotenoids (Olsson et al. 2008).

In birds and fish, dietary supplementation with tocopherols or other antioxidants enhanced carotenoid-based ornaments (Bertrand et al. 2006; Pike et al. 2007; Pérez et al. 2008). These findings were explained by a positive effect of antioxidants on carotenoid availability, either by protecting carotenoids from oxidation or by liberating carotenoids from antioxidant functions (Svensson and Wong 2011). In contrasts, the here-observed effects on γ-(β-) tocopherol concentrations were not mediated by changes in carotenoid availability, indicating that a different pathway may link tocopherol concentrations with coloration. One explanation is that tocopherols may protect ornamental carotenoids directly in the integument, preventing carotenoid bleaching and color fading (Hartley and Kennedy 2004). Alternatively, tocopherols may be related with coloration through other integumentary color components. The integument

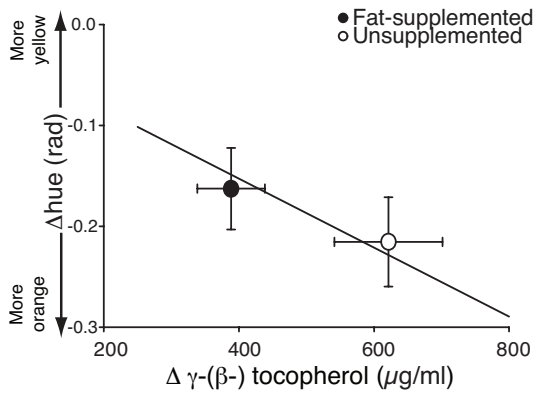

FIG. 6.—Relationship between changes in ventral hue and plasma γ-(β-) tocopherol concentration. Means (± SE) for fat-supplemented and unsupplemented groups are plotted. Lines reflect model predictions for the relationship between changes in ventral hue and plasma γ-(β-) tocopherol concentration.

of the common lizards, and of reptiles in general, consists of several structural and pigment components that may also determine coloration, like melanins and iridophores (Bagnara et al. 1968; Grether et al. 2004). The synthesis of melanin and purine crystals of iridophores depends on oxidative stress (Glantzounis et al. 2005; Galvan and Alonso-Alvarez 2008) and their reflective properties may therefore depend on antioxidants such as tocopherols.

Mate choice based on antioxidant-related ornaments may increase female reproductive success because antioxidant concentrations are important determinants of male fertility and survival (Catoni et al. 2008; Monaghan et al. 2009). In several lizard species, including the common lizard, males secrete tocopherol through femoral pores (Gabirot et al. 2008), which have been shown to affect female mate choice in European green lizards (Kopena et al. 2011). Females assess tocopherol content of femoral pore secretions and select males with

higher tocopherol concentrations (Kopena et al. 2011). Females may benefit from this selection because males with higher tocopherol concentrations are likely to be in better condition and defend better territories, which may ultimately improve female fitness (Kopena et al. 2011). Tocopherol availability may therefore influence the expression of different signals in different sensory modalities (visual and olfactory), suggesting that it may play a predominant role in intraspecific communication and mate choice in lizards (Candolin 2003).

In conclusion, we demonstrated that supplementation with dietary lipids negatively affected plasma carotenoid and tocopherol concentrations as well as reduced the expression of the ornamental coloration of male common lizards. Despite carotenoid supplementation significantly enhancing plasma lutein concentration, it had no effect on coloration and it did not offset the negative color effects of dietary lipids. Our study suggests that dietary lipids affected coloration through their effect on tocopherols and not through their effect on carotenoid plasma concentrations. This is a fundamental difference and suggests that ornamental coloration may provide a much more general criteria about individual health than previously thought. Our study is congruent with research on other lizard species, suggesting that color determination in lizards may importantly differ from birds and fish, where carotenoid availability governs color expression. Mate choice based on traits reflecting tocopherol availability may directly select for the evolution of food-selection strategies that maximize dietary intake of tocopherols in order to enhance antioxidant functions and improve ornamental traits (Catoni et al. 2008). Given that lipids are a major, essential nutrient, a conflict between carotenoid and tocopherol uptake and lipid intake may arise depending on the degree of covariation among these compounds in natural diets. If lipid access is independent of carotenoid and/or tocopherol access, as suggested by different studies (Barker et al. 1998; Finke 2002; Girling and Raiti 2004), competitive effects among nutrients could reinforce signal honesty, given that only animals in excellent condition may afford avoiding the ingestion of fat-enriched and, hence, highly energetic food in order to maximize carotenoid and tocopherol intake.

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Chapter V

Iridophores and not Carotenoids Account for Chromatic Variation of Carotenoid-based Coloration in Common Lizards (*Lacerta vivipara*)

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Abstract: Carotenoids need reflective background layers to shine. Such layers, consisting of leucophores and iridophores in fish, reptiles, and amphibians and of keratin- and collagen-derived structures in birds are generally assumed to show no or little environmental variability. However, recent studies suggest that this paradigm may not always hold true. Here, we investigate the origin of carotenoid-based ventral color variation of male common lizards (*Lacerta vivipara*) by dissecting among environmental variation derived from carotenoids and other integumentary components. In an *in vivo* experiment, we manipulated coloration by administrating lizards with corticosterone and dietary carotenoids. Treatments significantly affected the chromatic component of coloration by altering background-related reflectance and not carotenoid-related reflectance or skin carotenoid content. In an *in vitro* experiment, we show that altered background-related reflectance most likely results from changes in iridophore-based reflectance. Our findings thus demonstrate that carotenoid-based ornaments may not exclusively reflect differences in integumentary carotenoid content and, hence, in qualities linked to carotenoid deposition (*e.g.*, foraging ability or immune and antioxidant capacity), and they suggest that iridophores may instead reflect individual quality. Carotenoid-based coloration of male common lizards may be a multi-component signal, with iridophores reflecting environmental conditions and carotenoids reflecting different color morphotypes.

Key words: Carotenoid-based ornaments; Condition-dependent signaling; Honest signaling; Structural coloration.

Los Iridióforos y no los Carotenoides son Responsables de la Variación Cromática de la Coloración Basada en Carotenoides de la Lagartija de Turbera (*Lacerta vivipara*)

Resumen: Los carotenoides necesitan una capa reflectance de fondo para poder producir color. Se asume que tales capas, como los leucóforos e iridióforos de peces, reptiles y anfibios o las estructuras de queratina y colágeno de las aves, tienen poca o ninguna variabilidad debida al ambiente. Sin embargo, estudios recientes muestran que este paradigma no siempre se cumple. Aquí, investigamos el origen de la variación en la coloración ventral basada en carotenoides de los machos de lagartija de turbera (*Lacerta vivipara*) distinguiendo entre la variación ambiental resultante de los carotenoides y de otros componentes del integumento. En un experimento *in vivo* manipulamos la coloración de las lagartijas mediante la administración de corticosterona y de carotenoides. Los tratamientos afectaron de forma significativa el componente cromático de la coloración al alterar la reflectancia asociada al fondo y no la reflectancia asociada a carotenoides o al contenido de carotenoides en la piel. En un experimento *in vitro*, mostramos que, muy probablemente, la reflectancia asociada al fondo resulta de cambios en la reflectancia originada en los iridióforos. Nuestros resultados demuestran por tanto que los ornamentos basados en carotenoides puede que no reflejen de forma exclusiva las diferencias en el contenido de carotenoides del integumento y, por lo tanto, en las cualidades ligadas a la deposición de carotenoides (*e.g.*, abilidad para conseguir alimento, capacidad inmune o antioxidante), y sugieren que los iridioforos pueden ser los que, a su vez, reflejen la calidad individual. La coloración basada en carotenoides de los machos de lagartija de turbera puede ser una señal basada en múltiples componentes, con los iridióforos reflejando las condiciones ambientales y los carotenoides reflejando el polimorfismo de color.

Introduction

Carotenoid pigments are responsible for most of the bright yellow, orange, and red ornamental colorations of animals (Olson and Owens 1998). Carotenoid-based colorations usually function as condition-dependent signals that honestly reveal information about quality or condition of potential mates and competitors (Johnstone 1997). Signal honesty arises from the costs entailing the development of carotenoid-based ornaments (Zahavi 1975; Grafen 1990). Intense carotenoid-based colorations are costly to produce because ornamental incorporation of carotenoids is limited by **i.** dietary carotenoid availability (Hill 1994; Negro et al. 2000), given that animals cannot synthesize carotenoids *de novo* (Goodwin 1986), and by **ii.** carotenoid requirements of other physiological functions, like immune and antioxidant responses (Bendich 1993; Olson 1993; Chew and Park 2004) or vitamin A synthesis (Olson 1989). Individuals deposit carotenoids into ornamental integuments according to their quality to deal with these limitations (*e.g.*, foraging ability, health condition, or nutritional status; Hill 1990; Lozano 1994; von Schantz et al. 1999). Thus, variation in individuals' quality or condition that may result under different environmental conditions is assumed to induce changes in ornamental carotenoid deposition and, hence, in coloration (Andersson and Prager 2006).

Carotenoids have a purely pigmentary effect (*i.e.*, they absorb but do not emit visible light) and therefore need a reflective surface in order to produce color. Carotenoids responsible for coloration are located above other integumentary components whose reflective properties provide carotenoids with the necessary background reflectance. Reflective integumentary components include keratin- and collagen-derived matrices in birds (Prum 1999; Prum and Torres 2003) and iridophores, leucophores, and the collagen fascia in reptiles, fish, and amphibians (Bagnara and Hadley 1973; Grether et al. 2004). Because carotenoids absorb light exclusively in the shorter wavelengths of the visible spectrum (namely in the violet to blue wavelength range; Britton 1995), their integumentary incorporation leads to predominant reflection of longer wavelengths and thus to the typical yellow, orange, and red carotenoid-based colorations. Environmentally induced variation in integumentary incorporation of carotenoids therefore translates in chromatic variation given that it alters the ratio between short and long wavelength light (Shawkey and Hill 2005).

In contrast to the well-understood contribution of carotenoids to spectral reflectance, the contribution of integumentary components determining background reflectance and, more specifically, their susceptibility to environmental conditions has received little attention. Jacot et al. (2010) recently demonstrated that the intensity of the carotenoid-based plumage of great tits (*Parus major*) varies because of environmentally induced changes in the keratine-derived structure of feathers, providing evidence that other integumentary components besides carotenoids account for variation in carotenoid-based colorations. In great tits, keratin-derived structure reflects light uniformly after an in-

crease at short wavelengths, which results in white background reflectance and consequently in achromatic variation (Jacot et al. 2010). Thus, environmentally induced variation in the keratin-derived structure, affecting all wavelengths, and environmentally induced variation in carotenoid deposition, affecting only short wavelengths, additively contribute to the overall coloration (Jacot et al. 2010). However, some carotenoid-based ornaments posses a high reflectance peak at longer wavelengths that largely differs from the typical broad plateau of white backgrounds. The presence of such peaks indicates that integumentary components responsible for background reflectance produce color, *i.e.*, reflect certain wavelengths of visible light with more intensity than others (Prum and Torres 2003). Colored backgrounds, which occur in plumage (Siefferman et al. 2007; Griggio et al. 2010) and bare parts of birds (Prum and Torres 2003; Velando et al. 2006) and in the skin of fish (Grether et al. 2005), frogs (Richardson et al. 2009), and lizards (Macedonia et al. 2000; Vercken et al. 2007), imply that not only carotenoids but also other integumentary components may be responsible for chromatic variation of carotenoid-based ornaments (Fig. A1). Further, carotenoid deposition may not always depend on environmental conditions, as shown in species that exhibit discrete color morphotypes and where carotenoid-based ornaments are not environmentally plastic and do not function as condition-dependent signals (Dale 2000; Dale et al. 2001). Thus, environmentally induced chromatic variation may not necessarily result from variation in integumentary carotenoid content and, hence, it may not reflect differences among individuals in dealing with the cost of carotenoid deposition. Unraveling the contribution of integumentary components and whether they plastically respond to environmental parameters is therefore necessary for the understanding of the mechanisms underlying the expression of carotenoid-based ornaments, which forms the basis for understanding their evolution.

Here, we investigated whether other integumentary components than carotenoid pigments are responsible for flexible chromatic variation in carotenoid-based colorations. We investigated this question in male common lizards, *Lacerta vivipara*, which show carotenoid-based ventral coloration (Czeczuga 1980; Fitze et al. 2009). Male ventral coloration in *L. vivipara* reflects discrete color morphotypes (Sinervo et al. 2007) but it also shows chromatic variation in response to different environmental conditions (Meylan et al. 2007; Cote et al. 2008; Fitze et al. 2009; Cote et al. 2010). It has been repeatedly shown that carotenoid supplementation and enhanced circulating levels of carotenoids do not affect coloration (Fitze et al. 2009; San-Jose, et al. 2012a). The absence of this effect is consistent with discrete color morphotypes (Sinervo et al. 2007) although it contradicts studies showing environmental determination of male coloration. This incongruence may however indicate that some integumentary components are environmentally flexible whereas others may reflect male color morph. As in other lizard species, the integument of the common lizard is arranged in

contiguous chromatophore cell layers (Breathnach and Poyntz 1966; Bryant et al. 1967). Carotenoids are deposited in specific chromatophore cells; xanthophores and erythrophores, localized in the outermost layer of the dermis (Bagnara et al. 1968). A reflective second layer made up of iridophores is localized below this first layer, followed by an absorbing third layer of melanophores (Bagnara et al. 1968). The later two layers provide carotenoids with a background (Grether et al. 2004), which is colored as evidence by **i**. the presence of a high reflectance peak at long wavelengths (Fig. 1) and by **ii**. the extraction of carotenoids with acetone, which leads to blue ventral coloration (Fitze et al. 2009). This blue coloration suggests that the background more likely results from light reflectance in iridophores (Grether et al. 2004). Iridophores contain purine crystals arranged into stacked platelets, which produce color by constructive multilayer interference (Land 1972). Iridophores reflect light in a predominant wavelength range, which depends on the refractive index and size of the platelets, the refractive index of the cytoplasm present in the iridophores, and the spacing among platelets. Different platelet arrangements allow iridophores to produce different blue-green colors (Rohrlich and Porter 1972). Melanophores may amplify the reflective effect of iridophores by modulating the saturation of the blue-green color (for further details see Grether et al. 2004).

To investigate which integumentary components are responsible for environmental plasticity in the ventral coloration of *L. vivipara*, we ran an *in vivo* experiment in which we administrated lizards with corticosterone and two different carotenoid diets. Corticosterone has been shown to affect the chromatic component of the ventral coloration of *L. vivipara* (Fitze et al. 2009; Cote et al. 2010) but it is not clear whether the corticosterone effect originates from differential carotenoid incorporation into the integument. Moreover, it is not clear which part of the reflectance spectra is affected by corticosterone. We therefore used objective color measurements to disentangle variation in reflectance originating from carotenoids deposited in the integument from variation originating from changes in background components. To understand whether chromatic changes were the consequence of differential carotenoid deposition, we further investigated treatment effects on the integumentary carotenoid concentration as well as on the availability of carotenoids in blood and liver. Because carotenoids can be metabolized into vitamin A, we also investigated treatment effects in the hepatic concentration of vitamin A (Simpson 1983). We also tested which part of the reflectance spectra and, hence, which integumentary components determine color differences between male color morphotypes as well as whether color morphotypes differ in the amount of carotenoids deposited in the skin and other tissues.

To investigate whether iridophores can be responsible for carotenoid-independent chromatic changes, we ran an *in vitro* experiment in which we manipulated the osmotic environment of integumentary cells using different concentrations of phosphate-buffered saline (PBS). Changes in osmolarity have a direct effect on the intracellular spac-

ing of guanine platelets of iridophores (Bone and Denton 1971; Morrison et al. 1996), but do not affect carotenoid deposition. Increasing osmolarity reduces platelet spacing, which displaces peak wavelength reflectance towards shorter wavelengths and leads to less orange colorations (Lythgoe and Shan 1982; Morrison et al. 1996). In contrast, decreasing osmolarity augments platelet spacing, displacing peak wavelength reflectance towards longer wavelengths and thus producing more orange colorations (Land 1972).

Materials and Methods

Species Description

The common lizard, *Lacerta vivipara*, is a small ground dwelling lizard that inhabits peat bogs and moist heathlands across Eurasia (Sindaco and Jeremcenko, 2008). During maturation, males acquire a conspicuous white, yellow, or orange ventral coloration that ranges from the hind limbs to the throat (Sinervo et al. 2007). Carotenoids stored in xanthophores produce the observed yellow-orange coloration (Czeczuga 1980; Fitze et al. 2009). Moreover, extraction with acetone resulted in bluish skin colorations, which militates against the presence of non-carotenoid pigments like pteridines, which may be also responsible for yellow-orange colors (Fitze et al. 2009).

In the Pyrenees, common lizard males exhibit six color morphotypes determined by a putative single locus with three alleles (orange, *o,* yellow, *y*, and white, *w*; Sinervo et al. 2007). Males can be classified as putative homozygotes in the presence of a uniform orange (*oo*), yellow (*yy*) or white (*ww*) ventral coloration, or putative heterozygotes (*wo*, *wy*, *yo*) in the presence of mixed, mosaic-like coloration. Environmentally induced color changes have been demonstrated in relation to blood corticosterone levels (Fitze et al. 2009; Cote et al. 2010), population density (Meylan et al. 2007), population sex-ratio (Cote et al. 2008), parasite load, immune response, and oxidative stress (Cote et al. 2010). Color changes have been suggested to result from differential deposition of carotenoids in the integument (Fitze et al. 2009; Cote et al. 2010) but no effect of carotenoid supplementation on coloration has been demonstrated yet (Fitze et al. 2009).

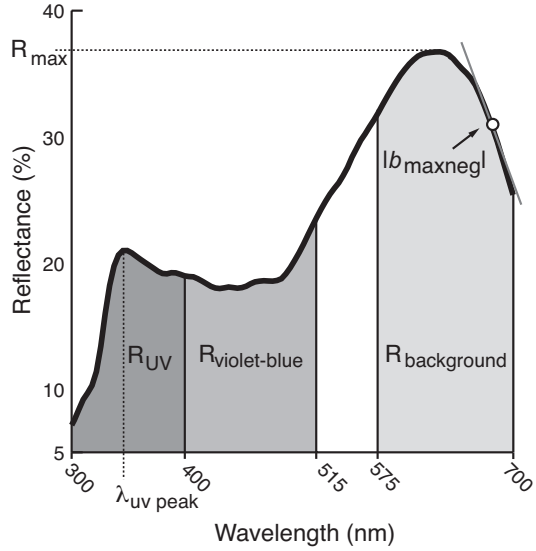


Fig. 1.—Average reflectance spectrum of the ventral coloration of male common lizards (N = 36). Spectral regions used for color parameter calculation are shaded and the location of peak reflectance in the ultraviolet part, of maximum negative slope (background slope) above 575 nm, and of peak reflectance above 575 nm are indicated.

Pre-experimental Methods

For the *in vivo* experiment, we collected 36 adult males from two neighboring populations at Somport (Central Pyrenees, Huesca, Spain, 42°47'N, 0°31'W). For the *in vitro* experiment, we collected 12 adult males from two Pyrenean populations; Somport and Puerto de Ibañeta (Western Pyrenees, Navarra, Spain, 43°1'N, 1°19'W). Lizards were brought to the laboratory at the Instituto Pirenaico de Ecología (Jaca, Huesca, Spain), where they were individually housed in *terraria* (25 × 15 × 15 cm) equipped with two shelters, a water pond, and peat soil as substrate. A 40 W bulb provided heat and light during a 10-h light:14-h dark photoperiod, and a UV light source provided both, UVB and UVA for two hours a day. We measured SVL (to the nearest 1 mm) and body mass (to the nearest 1 mg) and took a standardized photograph for posterior color morph determination by Dr. Barry Sinervo. We scored lizards as having 0, 1, or 2 putative *o* alleles (*O* scale), and 0, 1, or 2 putative *w* alleles (*W* scale; for further details see Sinervo et al. 2007).

In Vivo Experiment

Experimental Design.—Eight lizards were randomly assigned to a xanthophyll supplementation group (XAN), 8 to a β-carotene supplementation group (βCAR), 8 to a corticosterone administration group (CORT), and 12 to a control group (CONT). There were no significant differences among treatment groups in color parameters, SVL, body mass, body condition, color morph, date of capture, and population of origin (all $P > 0.05$). Lizards of the XAN group were fed with *Galleria mellonella* larvae injected with 0.03 mL of a solution of 200 mg lutein:zeaxanthin beadlets (5.58% lutein, 0.44% zeaxanthin; Hoffmann-La Roche, Basel, Switzerland) in 100 mL distilled water. Lizards in the βCAR group were fed with *G. mellonella* larvae injected with 0.03 mL of a solution of 160.45 mg β-carotene beadlets (7.5% β-carotene) and 39.55 mg control beadlets dissolved in 100 mL distilled water. Lizards in the CORT and CONT groups were fed with *G. mellonella* larvae injected with 0.03 mL of a solution of 200 mg control beadlets in 100 mL distilled water. Lizards in all groups were fed every two days for 14 days. If a lizard refused to eat, we left the larva in the *terrarium* and waited for one day before removing it.

The corticosterone treatment consisted of a daily application of 6.75 µg corticosterone diluted in 4.5 µL sesame oil. Lizards of the XAN, βCAR, and CONT group were treated with a control solution consisting of 4.5 µL of sesame oil only. Corticosterone and control solutions were applied on the lizard's back every night for 14 days (Meylan et al. 2003; Cote et al. 2006).

Tissue Sampling.—At the start of the experiment a blood sample was taken from the retro-orbital sinus of lizards using a heparinized microcapillary. Blood was centrifuged (5 min at 8900 g) and plasma stored at –80 °C until high performance liquid chromatography (HPLC) analyses. At the end of the experiment, we collected a second blood sample from a subset of 26 males (XAN: $N = 6$, βCAR: $N = 5$, CORT: $N = 6$, CONT: $N = 9$). Thereafter, lizards of the subsample were anaesthetized using 0.02 mL/g of a solution of

metomidine:ketamine (1:50) and decapitated. We extracted the ventral skin and the liver and rinsed them twice with PBS to remove blood traces and to avoid contamination with blood carotenoids. Finally, tissue samples were weighed (to the nearest 0.001 mg) and stored at –80 °C.

HPLC Analyses.—Concentrations of lutein, zeaxanthin, and β-carotene in plasma, skin, and liver as well as liver vitamin A_1 and vitamin A_2 concentrations were analyzed by HPLC using an adapted protocol of Olmedila et al. (1997). Plasma samples were mixed with 0.1 mL distilled water and 0.2 mL ethanol, vortexed, and extracted twice with 0.5 mL methylene chloride/hexane (1:5). Organic phases were pooled, evaporated to dryness, reconstituted (tetrahydrofuran; THF / ethanol; EtOH), and injected onto the HPLC column. Skin and liver samples were put into EtOH (25 min). Extraction was performed twice by ultrasound and intermittent vortex (5 min). Water (1 mL) and 2 mL methylene chloride/hexane (1:5) were added, vortexed, centrifuged, pooled, evaporated to dryness, reconstituted (THF / EtOH), and injected onto the HPLC column. Given the presence of carotenoid ester forms in the skin of common lizards (San-Jose et al. 2012b), skin samples were hydrolyzed following the protocol of Granado-Lorencio et al. (2001). The chromatographic system consisted of a Spheri-5-ODS column (Applied Biosystems, San Jose, CA, USA) with gradient elution of acetonitrile / methanol (85:15) for 5 min to acetonitrile / methylene chloride / methanol (70:20:10) for 20 min. Ammonium acetate (0.025 M) was added to the methanol. Compound detection was carried out by a photodiode array detector (model 2996, Waters Associates, Milford, MA) set at 450 nm for carotenoids and 326 nm for vitamin A_1 and A_2. Compound identification was carried out by comparing retention times with those of authentic standards and on-line UV-visible spectra.

In Vitro Experiment

One week after capture, lizards were euthanized by decapitation and ventral skin was removed and rinsed with phosphate-buffer saline (PBS). Skin was divided into three pieces, except for one small male where skin could only be divided into two pieces. One skin piece per male was assigned to a treatment of increasing osmolarity (hereafter referred to as IO group, $N = 12$ pieces), to a treatment of decreasing osmolarity (DO group, $N = 12$), or to a control treatment (CO group, $N = 11$). Skin pieces were first immersed in a standard PBS solution (295 mOsm/L). Thereafter, they were put into PBS solutions of increasing (1.5X, 442.5 mOsm/L; 2X, 590 mOsm/L; 3X, 885 mOsm/L) or decreasing osmolarity (0.5X, 147.5 mOsm/L; 0.25X, 73.75 mOsm/L; 0X, *i.e.*, distilled water, 0 mOsm/L), or, in the case of control pieces, to four different flasks of standard PBS solutions. Skin pieces remained in each solution for 20 min. Thereafter, we put them on a black photographic cloth and measured color using a photospectrometer.

Color Measurements

We measured coloration with a USB4000 spectrometer (Ocean Optics Inc., Dunedin, FL, USA), a standard reflection probe (QR400-7-UV/VIS-BX, Ocean Optics Inc.),

and a deuterium and tugsten-halogen light source (DT-MINI-2-GS, Ocean Optics Inc.) providing light between 300 and 700 nm. Using OOIbase software (Ocean Optics Inc.), reflectance of an area of approximately 0.12 cm^2 was measured (probe angle 45 °) in relation to a diffuse white standard (WS-1, Ocean Optics Inc.) and a dark reference.

We used the programs CLR and RCLR (Montgomerie 2008a; Montgomerie 2008b) to calculate several color parameters describing spectral intensity and spectral shape. To investigate effects on integumentary components responsible for background reflectance, we calculated absolute reflectance ($R_{background}$), maximum background reflectance (R_{max}), and background slope (absolute value of the maximum negative slope; $|b_{maxneg}|$). These parameters were quantified between 575 and 700 nm, which is outside the range where carotenoids absorb light (Fig. 1; Montgomerie 2006; Jacot et al. 2010). Hence, they exclusively reveal carotenoid-independent spectral variation resulting from other integumentary components (*i.e.*, melanophores and iridophores; Grether et al. 2004).

To investigate effects on integumentary carotenoid content, we calculated reflectance in the violet-blue range (violet-blue reflectance: $R_{400\text{-}515}$), where carotenoids absorb light (Shawkey et al. 2006). We also calculated absolute carotenoid chroma ($R_{violet\text{-}blue}$ / $R_{background}$). Absolute carotenoid chroma accurately reflects carotenoid content, if background components vary achromatically (*i.e.*, background components similarly affects violet-blue and background ranges; Jacot et al. 2010). However, in the presence of chromatic background components (*i.e.*, if background components differently affect violet-blue reflectance and reflectance above 575 nm), a worse correlation between absolute carotenoid chroma and integument carotenoid concentration is expected, because chromatic variation may not exclusively result from variation in carotenoid concentration (Fig. A1). We also calculated hue using Endler's (1990) segment classification method in order to make the results comparable with previous studies (Fitze et al. 2009; Cote et al. 2010).

To investigate effects on the UV spectra, we calculated reflectance in the UV range (300 to 400 nm; R_{UV}) and the spectral position of maximum reflectance in the UV range ($\lambda_{uv\ peak}$). Both parameters may be affected by background components and carotenoids (Macedonia et al. 2000; Jacot and Kempenaers 2007; Jacot et al. 2010).

During molt, ventral coloration becomes blurry and whitish until lizards finally shed their old skin (Bauwens et al. 1989). To account for this effect, we classified males according to their molt status as **i.** pre-molting, if they showed a blurry ventral coloration; or **ii.** non-molting, if they showed a normal bright coloration.

Statistics

Pearson's correlations between coloration and skin lutein, zeaxanthin, or β-carotene concentration were based on individuals of the CONT group of the *in vivo* experiment (N = 9) to avoid that correlations reflect differences between treatments rather than natural variation.

To test for treatment effects on coloration

(*in vivo* experiment), we fitted linear mixed-model ANCOVAS with lizard as a random factor. Starting models included measurement time (before / after experiment), treatment, and skin shedding status as factors, number of ingested larvae, *W* scale, and *O* scale as covariates, and all two-way interactions.

To analyze treatment effects on plasma lutein and zeaxanthin concentrations, we used linear mixed models with lizard as random factor and treatment and time (before / after experiment) as fixed factors, number of ingested larvae, *O* scale, and *W* scale as covariates, and their interactions. Because only small traces of β-carotene were detectable in common lizard plasma (San-Jose et al. 2012b), we tested whether its presence was time and treatment dependent using a logistic mixed model with lizard as random factor. Skin and liver concentrations of lutein, zeaxanthin, and β-carotene, and liver concentrations of vitamin A_1 and vitamin A_2 were analyzed using ANCOVAS. Full models included treatment as a factor and number of ingested larvae, *O* scale, and *W* scale as covariates, and their interactions.

Effects of the osmolarity treatment on coloration (*in vitro* experiment) were analyzed using mixed-model ANCOVAS with lizard and skin patch (nested within lizard) as random factors, and treatment (IO, DO, and CO groups), measurement time (number of solutions to which the tissue was exposed, *e.g.*, for the IO group: 1X = 1; 3X = 4), measurement time^2, and their interaction as fixed factors.

The statistical analyses were run using R 2.10.1 (R Development Core Team 2008) and JMPIN v.4 (SAS Institute Inc., Cary, NC). If necessary, variables were transformed to fulfill model assumptions and weighted least squares regressions were used if the homoscedasticity assumption was still violated (Neter et al. 1996). All models were simplified using backward elimination. The significance level was set at $P = 0.05$ (two-tailed). Multiple testing was accounted for following Hochberg procedure (Benjamini and Hochberg 1995).

Results

Relationships between Skin Carotenoid Concentration and Coloration

Skin β-carotene concentration negatively correlated with violet-blue reflectance ($r = -0.94$, $N = 9$, $P < 0.001$) and there existed a negative trend with absolute carotenoid chroma ($r = -0.62$, $N = 9$, $P = 0.08$). There was no significant correlation between skin β-carotene concentration and variables describing variation in background components and the UV part of the spectra (all $P > 0.12$; Table B1). No significant correlations existed between ventral coloration and skin lutein and zeaxanthin concentrations (all $P > 0.10$; Table B1).

Effects of Corticosterone and Carotenoid Supplementation on Coloration

Treatment induced significant changes in the reflectance spectra (Fig. 2). The temporal change in background coloration significantly differed among treatment groups (Table 1, treatment × time interaction). Lizards of the XAN, CONT, and CORT groups showed a significant decrease in background reflectance, maximum background reflectance and background slope, while no significant change existed in the βCAR group (Fig. 3A-C), indicat-

ing that no color change existed in the βCAR group and that in the other groups the reflectance peak in the long wavelengths became smaller and flatter (Fig. 2).

None of the treatments significantly affected violet-blue reflectance (Table 2, treatment × time interaction; Fig. 2, 4A). Absolute carotenoid chroma significantly increased in the XAN, CONT, and CORT groups, but not in the βCAR group (Table 2, Fig. 4B). Ventral hue significantly increased in the XAN group, which became more yellow (Table 2, Fig. 4C). A positive trend (P = 0.06) was found in the CONT group and no significant change existed in the βCAR and CORT groups (Fig. 4C). There were no significant treatment effects in any of the measured color parameters describing variation in the UV range (UV reflectance and the spectral location of the UV peak; Table 2).

Ventral coloration significantly depended on color morph and molt status (Tables 1, 2). Violet-blue reflectance significantly decreased with *O* scale (estimate ± SE = –4.15 ± 0.87 %) and there was a trend for UV reflectance (estimate ± SE = –1.52 ± 0.81 %). Absolute carotenoid chroma increased (estimate ± SE = 0.10 ± 0.06) and hue decreased (estimate ± SE = –0.08 ± 0.04) with increasing *W* scale. All other color parameters were not significantly affected by color scale, and interactions between color scale and treatment were not significant in any of the color parameters (all P > 0.1). Pre-molting lizards showed a significant decreased in background reflectance (estimate ± SE = –5.97 ± 1.55 %) and maximum background reflectance (estimate ± SE = –1.17 ±

Table 1.—Effects of corticosterone treatment and carotenoid supplementation on reflectance change of background components. Results stem from linear mixed model ANCOVAS with measurement time (pre-/post-experimental), treatment and molt status as fixed effects, and lizard as random factor.

Factor	F	d.f.	P	Explained variance (%)
Background reflectance ($R_{575\text{-}700}$)				
Time	12.74	1,27	0.001	2.80
Treatment × time	3.26	3,27	0.037	2.15
Molt status × time	11.57	1,27	0.002	2.55
Maximum background reflectance (R_{max})				
Time	18.18	1,27	< 0.001	4.08
Treatment × time	3.34	3,27	0.034	2.25
Molt status × time	13.25	1,27	0.001	2.98
Background slope ($\lvert b_{maxneg} \rvert$)				
Time	29.91	1,28	< 0.001	10.29
Treatment × time	4.44	3,28	0.011	4.58
Molt status × time	0.59	1,27	0.448	—

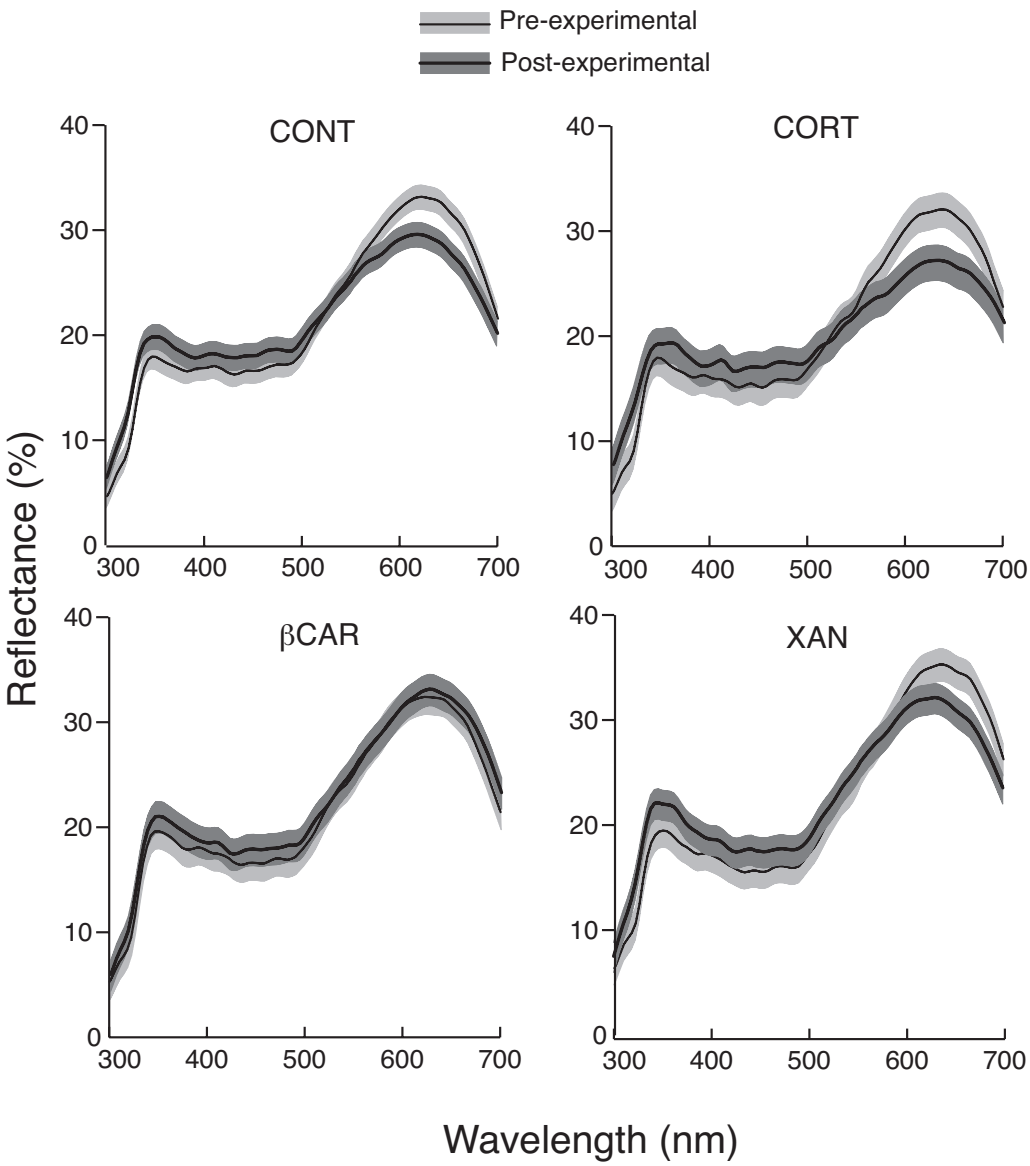


Fig. 2.—Mean ± SE (line and shading) of pre- and post-experimental reflectance spectra for CONT, CORT, βCAR, and XAN groups.

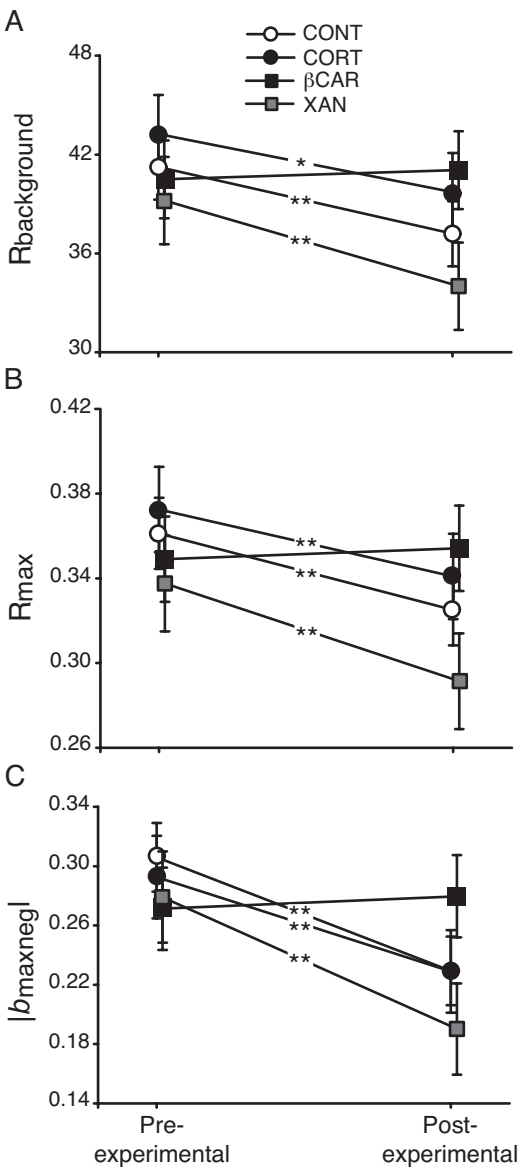


FIG. 3.—Treatment effects on changes in parameters reflecting variation in background components: (A) background reflectance, (B) maximum background reflectance, and (C) maximum background slope. Pre- and post-experimental means (± SE) are plotted. Asterisks indicate significant changes within treatment groups (** $P < 0.01$, * $P < 0.05$).

0.29%) and a significant increased in absolute carotenoid chroma (estimate ± SE = 0.16 ± 0.01) and hue (estimate ± SE = 0.17 ± 0.05), which indicates that, compared to non-molting lizards, their ventral coloration became less red and saturated.

Effects of Corticosterone and Carotenoid Supplementation on Carotenoid Concentration

At the start of the experiment, treatment groups did not significantly differ in lutein ($F_{3,19} = 1.26$, $P = 0.31$) and zeaxanthin plasma concentrations ($F_{3,15} = 0.72$, $P = 0.56$), and in β-carotene presence ($\chi^2 = 1.22$, d.f. = 3, $P = 0.75$). Treatment significantly affected the change in plasma lutein concentration (treatment × time: $F_{3,19} = 6.78$, $P < 0.01$). Lizards in the XAN group showed a significant increase in plasma lutein concentration (estimate ± SE = 7.8 ± 2.1 μg/mL, $t_{19} = 3.72$, $P < 0.01$) and no significant change existed in the βCAR ($t_{19} = 0.54$, $P = 0.59$), CONT ($t_{19} = 1.04$, $P = 0.50$), and CORT groups ($t_{19} = 2.39$, $P = 0.09$). Plasma zeaxanthin concentration significantly increased during the experiment (estimate ± SE = 0.016 ± 0.006 μg/mL, $F_{1,22} = 6.96$, $P = 0.02$), but this change was not significantly different among treatment groups (treatment × time: $F_{3,15} = 1.31$, $P = 0.31$). Plasma β-carotene presence did not significantly change over the course of the experiment and no treatment effect existed (time: $\chi^2 = 0.85$, d.f. = 1, $P = 0.36$, treatment × time: $\chi^2 = 1.48$, d.f. = 3, $P = 0.70$). The number of ingested larvae (all $P > 0.89$), color scales (all $P > 0.08$), and the interaction with treatment (all $P > 0.14$) did not significantly affect plasma carotenoid concentrations.

Table 2.—Effects of corticosterone treatment and carotenoid supplementation on parameters reflecting variation in the wavelength where carotenoids absorb light. Results stem from linear mixed model ANCOVAs with measurement time (pre-/post-experimental), treatment, and molt status as fixed effects, color scales as covariates, and lizard as random factor.

Factor	*F*	d.f.	*P*	Explained variance (%)
Violet-blue reflectance ($R_{400\text{-}500}$)				
Time	12.64	1,34	0.001	2.07
Treatment × time	0.69	3,15	0.570	—
Molt status × time	1.80	1,26	0.190	—
O scale	22.92	1,34	< 0.001	3.75
Absolute carotenoid chroma ($R_{400\text{-}500}$ / $R_{575\text{-}700}$)				
Time	35.44	1,26	< 0.001	7.85
Treatment × time	3.10	3,26	0.044	2.06
Molt status × time	16.30	1,26	< 0.001	3.61
W scale	17.30	1,26	< 0.001	3.83
Hue				
Time	4.79	1,26	0.038	1.11
Treatment × time	3.41	3,26	0.032	2.37
Molt status × time	12.51	1,26	0.002	2.90
W scale	5.09	1,26	0.033	1.18
UV reflectance ($R_{300\text{-}400}$)				
Time	16.66	1,34	< 0.001	4.66
Treatment × time	0.94	3,14	0.445	—
Molt status × time	3.49	1,27	0.073	—
O scale	3.54	1,34	0.068	0.99
UV peak ($\lambda_{UV\ max}$)				
Time	0.47	1,34	0.499	—
Treatment × time	0.48	3,17	0.703	—
Molt status × time	0.42	1,20	0.526	—

Treatment significantly affected skin lutein and zeaxanthin but not skin β-carotene concentration (Table 3, Fig. 5A-C). The XAN group showed higher lutein concentrations than the other treatment groups and higher zeaxanthin concentrations than the βCAR and the CONT groups (Fig. 5A-C). Skin lutein and zeaxanthin but not β-carotene concentration increased with the number of ingested larvae (lutein: estimate ± SE = 0.062 ± 0.03

µg/mg; zeaxanthin: estimate ± SE = 0.017 ± 0.008 µg/mg; Table 3) and no significant interaction existed between treatment and the number of ingested larvae ($P > 0.3$). Skin zeaxanthin and β-carotene concentrations decreased with increasing *W* scale (zeaxanthin: estimate ± SE = –0.019 ± 0.003 µg/mg, β-carotene: estimate ± SE = –0.001 ± 0.0002 µg/mg). Skin lutein concentration was not affected by color scale (Table 3) and there were no significant interactions between treatment and color scales and between color scales (all $P > 0.31$).

The hepatic concentration of lutein, zeaxanthin, and β-carotene significantly differed between treatment groups (Table 3). Higher lutein and β-carotene concentrations existed in the XAN group than in the other treatment groups. Zeaxanthin concentration of the XAN group was significantly higher than in the βCAR group, but did not differ from the CORT and CONT groups (Fig. 5D-F). Hepatic β-carotene but not hepatic lutein and zeaxanthin concentration significantly increased with the number of ingested larvae (estimate ± SE = 0.006 ± 0.002 µg/mg; Table 3). Hepatic lutein and zeaxanthin concentration increased with *W* scale (lutein estimate ± SE = 4.85 ± 1.36 µg/mg; zeaxanthin estimate ± SE = 0.593 ± 0.14 µg/mg), whereas β-carotene concentration decreased with increasing *O* scale (estimate ± SE: –0.019 ± 0.005 µg/mg; Table 3). No significant interactions existed between the color scales and treatment and between color scales (all $P > 0.87$).

Treatment did not significantly affect the hepatic vitamin A_2 concentration ($F_{3,19} = 0.06$, $P = 0.98$) but it significantly affected the hepatic vitamin A_1 concentration ($F_{3,22} = 3.10$, $P = 0.045$). Vitamin A_1 concentration was significantly higher in the βCAR than in the

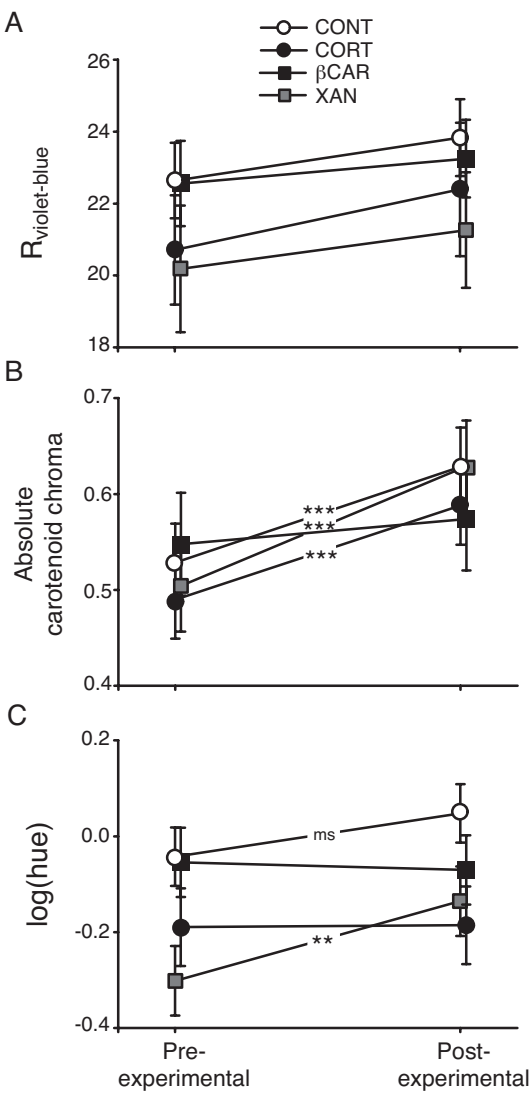
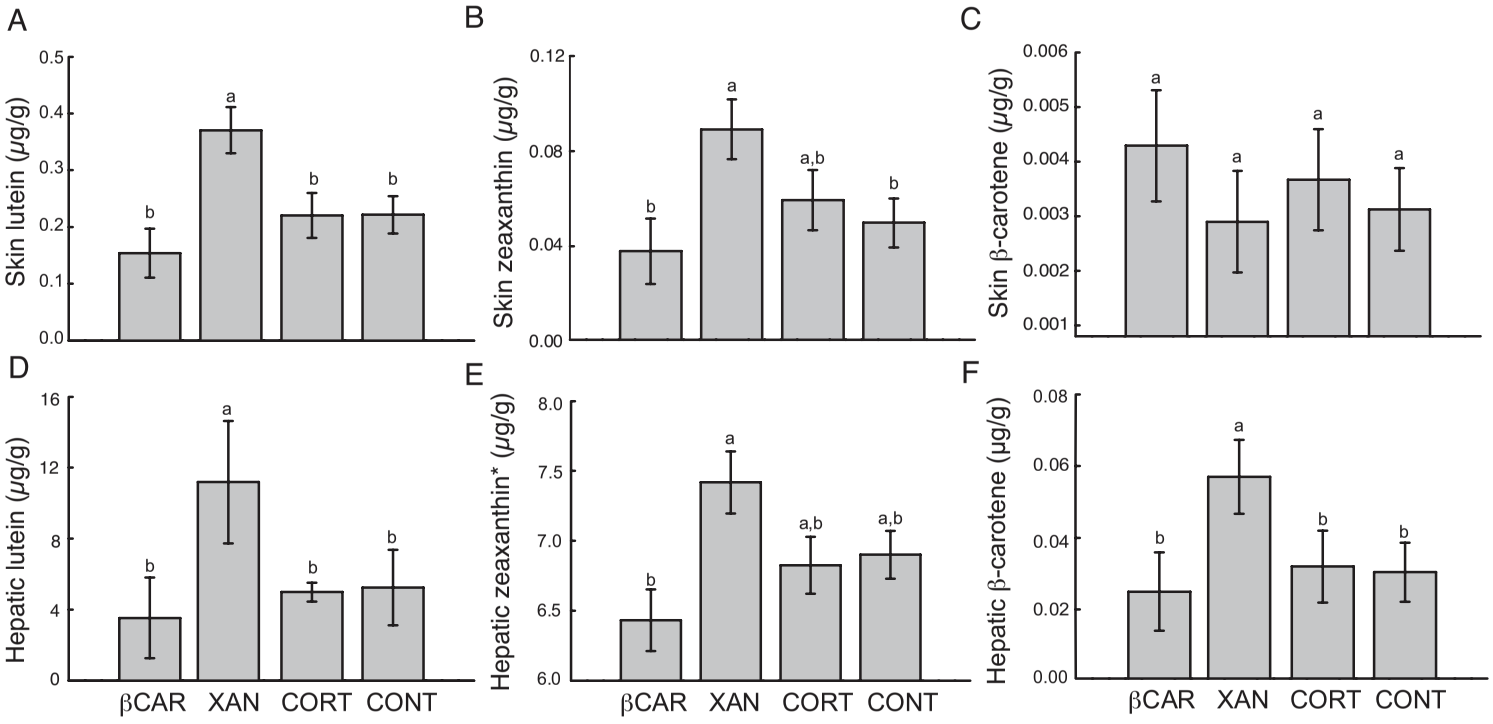


FIG. 4.—Treatment effects on changes in parameters reflecting variation in the wavelength where carotenoids absorb light: (A) violet-blue reflectance, (B) absolute carotenoid chroma, and (C) hue. Pre- and post-experimental means (± SE) are plotted. Asterisks indicate significant changes within treatment groups (*** $P < 0.001$, ** $P < 0.01$, ms (marginally significant) $P = 0.06$).

TABLE 3.—Effects of treatment, color scales, and number of ingested larvae on skin and liver concentrations of lutein, zeaxanthin, and β-carotene. Given are results from ANCOVAS using backward elimination ($N = 26$).

Factor	β-Carotene				Lutein				Zeaxanthin			
	F	d.f.	*P*	Explained variance (%)	*F*	d.f.	*P*	Explained variance (%)	*F*	d.f.	*P*	Explained variance (%)
Skin												
Treatment	0.68	3,21	0.73	—	4.76	3,20	0.01	30.48	3.35	3,20	0.04	24.05
W scale	8.64	1,24	< 0.01	26.48	< 0.01	1,19	0.98	—	5.55	1,20	0.03	13.28
O scale	0.11	1,19	0.75	—	3.72	1,20	0.07	10.23	0.25	1,19	0.62	—
Ingested larvae	0.32	1,20	0.58	—	4.79	1,20	0.04	8.00	5.39	1,20	0.03	12.90
Liver												
Treatment	5.94	3,20	< 0.01	10.36	3.14	3,21	0.047	22.02	3.28	3,21	0.04	22.13
W scale	0.70	1,19	0.41	—	16.04	1,21	< 0.01	37.54	16.6	1,21	< 0.01	37.62
O scale	45.60	1,20	< 0.01	26.46	0.14	1,19	0.71	—	< 0.01	1,19	0.99	—
Ingested larvae	8.03	1,20	0.01	4.66	2.20	1,20	0.15	—	2.49	1,20	0.13	—



* square-root transformed

FIG. 5.—Treatment effects on skin and hepatic concentrations of lutein, zeaxanthin, and β-carotene (mean ± SE). Different letters indicate statistically significant differences.

CONT (estimate ± SE = 0.18 ± 0.05 µg/mg, t_{22} = 3.32, P = 0.019) and CORT groups (estimate ± SE = 0.16 ± 0.06 µg/mg, t_{22} = 2.69, P = 0.039) and tended to be higher than in the XAN group (estimate ± SE = 0.13 ± 0.06 µg/mg, t_{22} = 2.30, P = 0.063). The hepatic vitamin A_1 and vitamin A_2 concentrations did not significantly depend on color scales (all P > 0.25), their interactions (P > 0.5), their interaction with treatment (all P > 0.64), and the number of ingested larvae (all P > 0.78).

Effects of Osmolarity on Coloration

Changes in osmolarity significantly affected the shape of the reflectance spectra (Fig. B1). No significant differences existed between osmolarity treatment groups in color parameters at the first measurement, where all skin pieces were immersed in standard 1X solutions (all P > 0.1). Changing osmolarity induced significant changes in all color parameters (treatment × time interactions in Table 4, Fig. 6). Background reflectance significantly decreased over time in the IO group (time: t_{31} = 12.89, P < 0.01) and significantly increased in the CO group (time: t_{31} = 3.04, P < 0.01) whereas no significant change existed in the DO group (time: t_{29} = 1.83, P = 0.10; t > 3.66, P < 0.01 for all pairwise treatment × time contrasts). In the IO group, maximum background reflectance increased from 1X to 1.5X solutions and decreased from 1.5X to 3X solutions (time: t_{31} = 7.48, P < 0.01; time^2: t_{31} = 5.08, P < 0.01; Fig. 6B). In the CO group maximum reflectance significantly increased over time (time: t_{31} = 3.60, P < 0.01), while no significant change existed in the DO group (time: t_{28} = 1.72, P = 0.20; t > 3.43, P < 0.01 for all pairwise treatment × time contrasts). In the IO group, background slope increased from 1X to 1.5X and then decreased (time: t_{31} = 5.65, P < 0.01; time^2: t_{31} = 6.69,

TABLE 4.—Effects of osmolarity treatments on ventral coloration. Results of mixed model AN-

Factor	F	d.f.	P
Background reflectance ($R_{575\text{-}700}$)			
Time	55.42	1,90	< 0.001
Treatment	17.45	2,90	< 0.001
Time × treatment	81.31	2,90	< 0.001
Maximum background reflectance (R_{max})			
Time	15.43	1,87	< 0.001
Time^2	20.39	1,87	< 0.001
Treatment	0.42	2,87	0.661
Time × treatment	36.67	2,87	< 0.001
Time^2 × treatment	10.20	2,87	< 0.001
Background slope ($\lvert b_{maxneg} \rvert$)			
Time	70.14	1,87	< 0.001
Time^2	0.02	1,87	0.901
Treatment	49.50	2,87	< 0.001
Time × treatment	27.16	2,87	< 0.001
Time^2 × treatment	39.94	2,87	< 0.001
Violet-blue reflectance ($R_{400\text{-}500}$)			
Time	67.05	1,90	< 0.001
Treatment	3.54	2,90	0.033
Time × treatment	11.04	2,90	< 0.001
Absolute carotenoid chroma ($R_{400\text{-}500}$ / $R_{575\text{-}700}$)			
Time	223.04	1,90	< 0.001
Treatment	10.03	2,90	< 0.001
Time × treatment	68.55	2,90	< 0.001
Hue			
Time	96.89	1,90	< 0.001
Treatment	37.17	2,90	< 0.001
Time × treatment	62.73	2,90	< 0.001
UV reflectance ($R_{300\text{-}400}$)			
Time	46.44	1,90	< 0.001
Treatment	8.19	2,90	< 0.001
Time × treatment	21.87	2,90	< 0.001
UV peak ($\lambda_{UV\ max}$)			
Time	20.38	1,90	< 0.001
Treatment	4.29	2,90	0.017
Time × treatment	3.65	2,90	0.030

$P < 0.01$; Fig. 6C), while in the DO group a decrease from 1X to 0.25X was followed by a slight increase from 0.25X to 0X (time: $t_{31} = 9.31$, $P < 0.01$; time^2: $t_{31} = 5.38$, $P < 0.01$). No significant change was observed within the CO (time: $t_{30} = 0.56$, $P = 0.58$; $t > 2.19$, $P < 0.05$ for all pairwise treatment × time contrasts).

Violet-blue reflectance significantly increased in all treatment groups (all $P < 0.01$; Table 4, Fig. 6D and Fig. B1). This increment was significantly higher in the DO group compared to the IO ($t_{90} = 5.80$, $P < 0.001$) and CO groups ($t_{90} > 5.80$, $P < 0.01$), and it did not significantly differ between the IO and CO group ($t_{90} = 0.14$, $P = 0.89$). Absolute carotenoid chroma and hue significantly increased in the IO group ($t_{30} = 12.36$, $P < 0.01$ and $t_{30} = 10.80$, $P < 0.01$ respectively) and in the DO group ($t_{29} = 11.00$, $P < 0.01$; $t_{29} = 3.53$, $P < 0.01$) and no significant change was found in the CO group ($t_{30} = 0.41$, $P = 0.68$; $t_{30} = 1.58$, $P = 0.12$; Fig. 6E-6F). The observed increment was significantly higher in the IO group than in the DO group for both absolute carotenoid chroma ($t_{90} = 2.51$, $P = 0.014$) and hue ($t_{90} = 4.37$, $P < 0.001$).

UV reflectance significantly increased over time in the DO ($t_{29} = 7.64$, $P < 0.01$) and CO group ($t_{31} = 3.70$, $P < 0.01$) and no significant change existed in the IO group ($t_{30} = 0.12$, $P = 0.91$; $t > 2.56$, $P < 0.05$ for all pairwise treatment × time contrasts; Fig. 6G). There was a significant in increase in the spectral position of the UV peak (shift towards longer wavelength) in the IO group compared to the CO group ($t_{90} = 2.84$, $P < 0.01$) and no difference existed between the IO and DO group ($t_{90} = 1.43$, $P = 0.15$; Fig. 6H). No significant differences existed between the DO group and the CO group ($t_{90} = 1.31$, $P = 0.19$).

Discussion

In many species, dietary administration of carotenoids enhances carotenoid deposition in the integument (McGraw et al. 2002a; McGraw et al. 2004), which leads to changes in short but not in long-wavelength reflectance and thereby in the chromatic component of carotenoid-based ornaments (Shawkey and Hill 2005; Shawkey et al. 2006; Jacot et al. 2010). Here, we demonstrated that differential β-carotene intake accounts for chromatic differences in male common lizards. However, the chromatic differences did not result from differences in the integumentary deposition of β-carotene, as revealed by the HPLC analysis. Spectral differences between lizards supplemented with β-carotene and the other treatment groups exclusively occurred at long wavelengths (*i.e.*, in background reflectance, maximum background reflectance, and in background slope) and, hence, in the range where carotenoids do not absorb light. These results therefore indicated that integumentary components, which provide carotenoids with a reflective background, and not carotenoids were responsible for the chromatic differences observed between the β-carotene supplemented group and the other treatment groups.

As previously demonstrated (Fitze et al. 2009; Cote et al. 2010), ventral coloration of corticosterone-treated lizards was more orange (lower hue values) at the end of the experiment compared to control and xanthophyll-supplemented lizards (Fig. 4C). Hue differ-

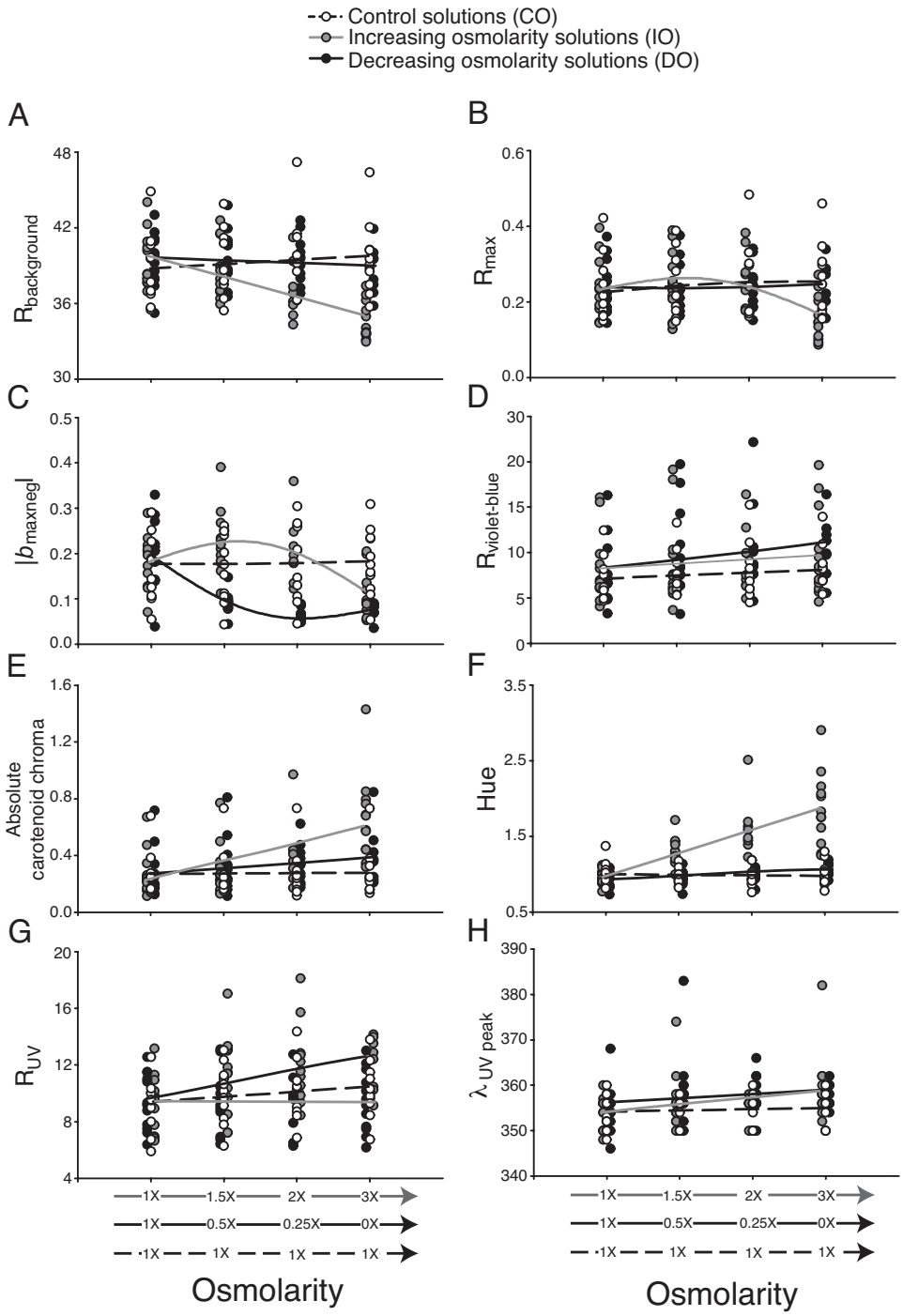


FIG. 6.—Effects of osmolarity on the measured color parameters. Given are observed values and model predictions (lines). Different colors indicate the three osmolarity treatment groups. Abscises reflect subsequent measurements and the applied osmolarity for each measurement and treatment group.

ences were the result of differences between treatment groups in yellow (550-625 nm) and red segment reflectance (625-700 nm; see Endler's segment classification method; formula 17 in Endler 1990). In corticosterone-treated lizards, reflectance decreased similarly in the yellow and red segments (similar reflectance decrease at both sides of the long-wavelength peak; Fig. 2), which according to the segment classification method leads to no change in hue. On the contrary, in the xanthophyll-supplemented and control group, reflectance decreased more in the red segment than in the yellow segment (higher decrease on the right side of the peak; Fig. 2), which leads to more yellow hue values. This change in spectral shape was more evident in xanthophyl-supplemented lizards (Fig. 2), where significant differences in hue change existed, than in control lizards, where a tendency was found. This shows that hue differences between corticosterone-treated lizards and control and xanthophyl-supplemented lizards resulted from differences in long wavelength reflectance and not from differences in short wavelength reflectance, which indicates that carotenoids did not account for this chromatic change. This finding, together with the fact that no effect of corticosterone administration on carotenoid concentrations of skin and other tissues was found, indicates that other integumentary components, *i.e.*, iridophores or melanophores (Breathnach and Poyntz 1966; Bryant et al. 1967; Grether et al. 2004) were responsible for this chromatic change.

Melanophores generally provoke achromatic changes but when located below xanthophores, erythrophores, and iridophores, they may induce chromatic changes (Grether et al., 2004). In this situation, melanophores absorb more light at wavelengths where light is less effectively absorbed by xanthophores and erythrophores and where light is less effectively reflected by iridophores (for further details see Fig. 13 in Grether et al., 2004). Decreased absorbance in the melanophore layer (*e.g.*, through decreased melanin content or dispersion) induces an increase in reflectance that is more apparent at longer wavelengths, which leads to a flatter background slope and to no or a positive change in maximum reflectance. In contrast, increased absorbance in the melanophore layer leads to a decrease in reflectance, especially at longer wavelengths, and hence to an increase in background slope and to no or a negative change in maximum reflectance (Grether et al., 2004). In our experiment, spectral changes were not congruent with chromatic changes expected from changes in the melanophore layer. Here, we observed a decrease in maximum background reflectance together with a decrease in background slope, which goes against the above-outlined predictions and which may therefore discard that melanophores accounted for the chromatic variation observed in the *in vivo* experiment.

Our results were however in line with the predictions from changes in iridiophore reflectance (Grether et al., 2004). Iridophores contain sacks of finely arranged crystal platelets, which allows them to selectively reflect visible light (*i.e.*, produce color) by a phenomenon of constructive multilayer interference that depends on the relationship between crystals size

and distance between crystals (Land 1972; Fujii 1993). By manipulating skin osmolarity and, hence, crystal spacing, we demonstrated that iridophores may lead to important chromatic changes. With increasing osmolarity coloration became less saturated and less orange, as predicted from decreased crystal spacing due to hyperosmotic conditions (Lythgoe and Shan 1982; Morrison et al. 1996). More specifically, increasing osmolarity induced spectral changes at long wavelengths (*i.e.*, a decrease in background reflectance and maximum background reflectance, and a concave change in background slope). Decreasing osmolarity did not increase background reflectance as expected from an increase in crystal spacing (Land 1972). Contrarily, decreasing osmolarity reduced long-wavelength reflectance and increased short-wavelength reflectance (UV and violet-blue reflectance). In addition to increase crystal spacing, increased cell volume because of hyposmotic solutions may induce free movement of crystal platelets, which alters platelet orientation and leads to less regular crystal arrangement (Bone and Denton 1971). In these circumstances, decreasing osmolarity impairs constructive interference of light and thereby the degree with which light is selectively reflected by iridophores. As a consequence, iridophore-based reflectance becomes more homogeneous and, as observed here, it increases at wavelengths of lower reflectance (*i.e.*, in the UV and violet-blue ranges) and decreases at wavelengths of higher reflectance (*i.e.*, above 550 nm; Fig. B1 panel B; Huxley 1968). Results from the *in vitro* experiment show that iridophores determine the relative contribution of short and long wavelengths to the overall spectrum. More specifically, iridophores determined the height and shape of the reflectance peak located at long wavelengths and, hence, the color parameters that were affected in the *in vivo* experiment. The diminishment in maximum background reflectance and maximum background slope observed in the *in vivo* experiment are in line with the diminishment in maximum background reflectance and maximum background slope observed in high osmolarity solutions (3X, Fig. 6) and hence with a decrease in crystal spacing. This indicates that changes in crystal spacing might have been responsible for the differences observed between the treatment groups of the *in vivo* experiment and, hence, that iridophores accounted for the observed chromatic changes.

Taken together, our findings indicate that iridophores are flexible integumentary components able to induce chromatic variation in response to environmental factors, which further suggests that they could be the basis for condition-dependent signaling in *L. vivipara*. Although the exact mechanisms modulating iridophore reflectance are not fully understood, it has been suggested that they rely on hormonal and neural control (Rohrlich and Porter 1972; Cooper and Greenberg 1992; Mäthger and Hanlon 2007; Mills et al. 2008). Hormonal and neural controllers may modulate iridophore reflectance by inducing contractile activity of the actin filaments that link successive crystals in parallel stacks (Rohrlich 1974). Direct effects of β-carotene on iridophores are unlikely, given the lack of differences in skin β-carotene concentrations between treatment groups. However, indirect

effects may exist. β-Carotene supplementation led to increased hepatic vitamin A_1 concentrations, which indicates that supplemented β-carotene was used for the synthesis of vitamin A_1 (Simpson 1983). Vitamin A_1 controls the ontogenetic development of iridophores (Miwa and Yamano 1999; Bolker and Hill 2000) and it is involved in several hormonal routes (Edem 2009). This suggests that β-carotene intake may indirectly affect iridophore-based reflectance through its effects on vitamin A_1 and that it may have prevented color fading in β-carotene supplemented lizards. However, disentangling between the different mechanisms is out of the scope of this article and needs to be the subject of future studies.

In contrast to iridophore-based reflectance, reflectance depending on integumentary carotenoids showed no environmental flexibility, which is in clear contrast to the general belief that integumentary deposition of carotenoids mainly determines the environmental component of carotenoid-based signals (Badyaev and Hill 2000). To date, all studies manipulating dietary carotenoid intake in lizards have found no significant effect of dietary carotenoid supplementation on coloration (Olsson et al. 2008; Fitze et al. 2009; Steffen et al. 2010). This suggests that integumentary carotenoid deposition may be differently controlled in lizards than in birds and fish. Our results further demonstrated that xanthophyl intake leads to increased xanthophyll concentrations in blood, hepatic reserve tissues, and even in the skin, without inducing any measurable effect on coloration. This finding suggests that factors different from xanthophyll availability may ultimately control xanthophyll incorporation into erythrophores and xanthophores. Here, we found that the skin concentration of some carotenoids was negatively correlated with the number of white alleles, indicating that the skin carotenoid content was higher in the yellow and orange and, hence, more colorful morphotypes. Further, the number of orange alleles negatively correlated with reflectance in the violet-blue range, suggesting that orange males incorporate more carotenoids into the integument. This suggests that integumentary carotenoids are linked to male color morph and the associated life-history strategies, and that tight genetic determination of carotenoid incorporation may explain the absence of environmental effects on integumentary carotenoid incorporation (Sinervo and Zamudio 2001; Svensson et al. 2001; Huyghe et al. 2010). Different components in the integument of the common lizard may therefore convey different information about individual phenotype; carotenoids may reflect the genetic context whereas iridophores may reflect condition-dependent variation. Thus, ventral carotenoid-based coloration of common lizards may be a multiple-message signal where a single signal, perceived coloration, provides different messages (Grether et al. 2004; Vercken et al. 2008).

In conclusion, our study showed that environmentally induced color variation in carotenoid-based ornaments does not necessarily result from differences in integumentary carotenoid content. The observed condition-dependent chromatic changes were in line with changes in iridophore platelet arrangement. To our knowledge, this experimentally

demonstrates for the first time that other integumentary components than carotenoids account for condition-dependent chromatic variation of carotenoid-based ornaments. This has important implications for the understanding of the evolution of carotenoid-based signals because chromatic differences in carotenoid-based colorations do not always reflect differences in carotenoid availability and carotenoid use and, thereby, in individual quality related to carotenoids (*e.g.*, foraging ability, immune response, antioxidant capacity). As shown here, carotenoid-based colorations may function as multiple-message signals where different integumentary components reveal different aspects of individual phenotype. Our findings indicate that chromatic variation in carotenoid-based coloration needs to be carefully interpreted in animal groups with iridophores in their integument (*i.e.*, fish and amphibians; Bagnara 1966; Bagnara et al. 1968) but also in other animal groups like birds where keratin- and collagen-derived reflective structures may also contribute to chromatic variation (Keyser and Hill 1999; Keyser and Hill 2000; McGraw et al. 2002b; Prum and Torres 2003; Prum 2006). Our study therefore highlights that special caution needs to be applied when deriving evolutionary theories about signal content from chromatic variation and it suggests that only detailed studies investigating which integumentary components are responsible for observed variation in reflectance spectra may allow deriving more general conclusions.

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APPENDIX A: Spectral changes in carotenoid-based ornaments

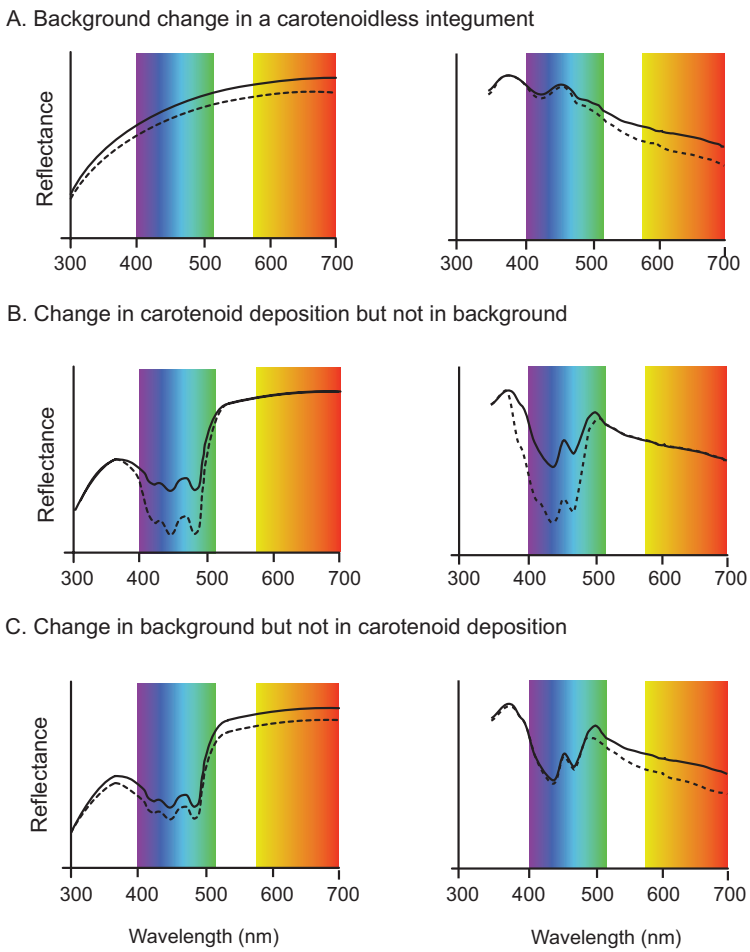

FIG. A1.— Predicted chromatic and achromatic changes in carotenoid-based ornaments with different background reflectance. Depicted are the ranges used to measure violet-blue reflectance (400-515 nm) and background reflectance (575-700 nm). (A) Reflectance change predicted for a white background reflectance (left chart, for further details see Jacot et al. 2010) and for a blue background (right chart, for further details see Grether et al. 2004) when carotenoid are absent. (B). In integuments with white and blue background reflectance (left and right charts, respectively), increased carotenoid deposition leads to decreased violet-blue reflectance, which induces chromatic variation by increasing the predominance of long wavelength reflectance. (C). In integuments with white background (left chart), decreased background reflectance leads to changes in violet-blue reflectance and background reflectance, which exclusively induces achromatic variation given that the relative contribution of short and long wavelength reflectance remains unchanged. In integuments with blue background (right chart), decreased background reflectance induces chromatic variation by affecting long wavelength reflectance more than short wavelength reflectance.

APPENDIX B: Supplementary results

TABLE B1.—Pearson's correlations between color variables and skin carotenoid concentration ($N = 9$).

	β-Carotene		Lutein		Zeaxanthin	
Color variable	*r*	*P*	*r*	*P*	*r*	*P*
$R_{background}$	–0.18	0.64	–0.20	0.60	0.06	0.88
R_{max}	–0.09	0.82	–0.30	0.43	–0.01	0.97
$\lvert b_{maxneg} \rvert$	–0.16	0.68	0.52	0.15	0.13	0.75
$R_{violet\text{-}blue}$	–0.94	<0.001	0.42	0.26	0.46	0.21
Absolute carotenoid chroma	–0.62	0.08	0.58	0.10	0.43	0.24
Hue	0.27	0.49	0.06	0.87	<0.01	0.99
R_{UV}	–0.56	0.12	0.17	0.66	0.43	0.24
$\lambda_{UV\ peak}$	–0.27	0.48	–0.05	0.90	–0.24	0.53

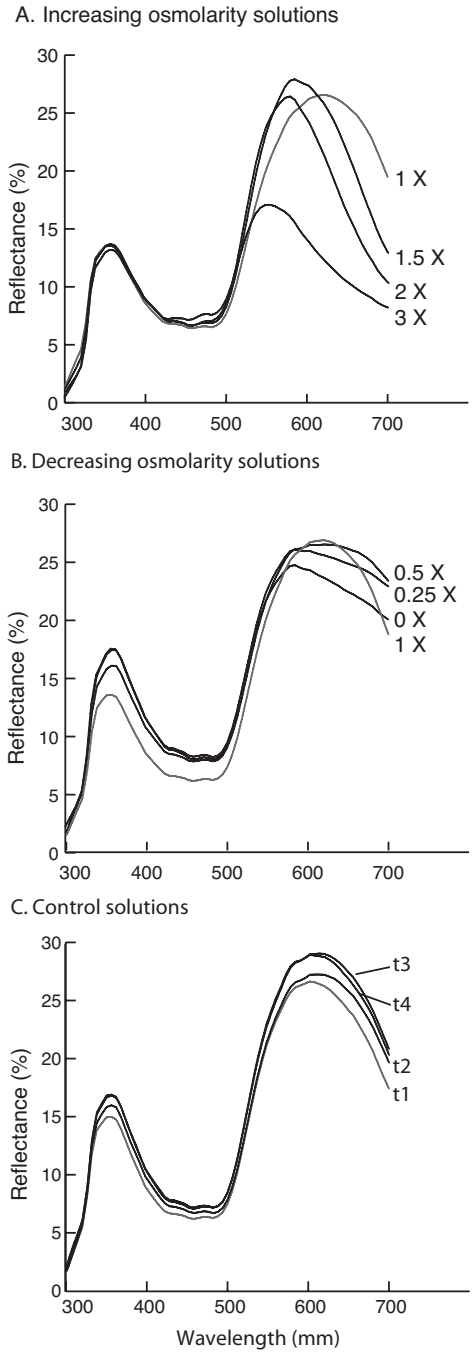

FIG. B1.—Average reflectance spectra of the four treatment levels of (A) the increasing osmolarity treatment (IO) and (B) the decreasing osmolarity treatment (DO), and (C) for each of four successive control solutions (1X; CO). Depicted in grey are measured reflectance spectra of skin peaces immersed in a standard 1X solution, at the start of the experiment.

Chapter VI

Experimental Evidences for Negative Frequency-dependent Sexual Selection and Morph-dependent Juvenile Survival as Drivers of Rapid Rock-paper-scissors Cycles of Male Colour Morphs

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Abstract: Frequency-dependent selection (FDS) acting on colour polymorphism and associated alternative reproductive tactics may lead to the appearance of complex rock-paper-scissors (RPS) dynamics, where the frequency of three distinct colour morphs cycles alternating the dominance of each strategy in the population. Recently, it has been described the occurrence of rapid RPS cycles of the orange, white, and yellow male morphs of the common lizard, *Lacerta vivipara*. However, common lizards present certain biological features; long life span and marked age classes that are suggested to lengthen RPS cycles by hindering generational replacement and, hence, annual changes in colour morph frequency. In order to reconcile the observed rapid RPS cycles of the common lizard with the theoretical conditions under which RPS dynamics are expected to evolve, it has been theorized that RPS dynamics of the common lizard should be driven by **i.** female context-dependent choice of morph mates and by **ii.** frequency-dependent recruitment of juvenile morphs. We tested these predictions using six seminatural populations of common lizards, where we manipulated the morph frequency of male adult and yearling cohorts to reproduce two different phases of the RPS cycle; three populations going to high white (*w*) frequency and three populations going to high orange (*o*) frequency. We found that male mating success depends on male morph and the established RPS phase, with *o* males having a higher mating success in populations going to high *w* frequency and yellow (*y*) males having a higher mating success in populations going to high *o* frequency. We further demonstrated that juveniles sired by morph with the highest mating success in each RPS phase survived better than juveniles sired by the rest of the morphs in the populations. All together, our findings support that colour morphs in the common lizard are subjected to FD sexual and natural selection. Given that male mating success was congruent with the expected future morph frequency (*i.e.*, with the yearling morph frequency) and not with the current adult frequency, our study supports that FD sexual selection of male morphs is driven by female context-dependent mate choice. Female may assess yearling morph frequency as a proxy of the future RPS frequency that offspring will experience, mating sire morphs that may enhance offspring fitness in such future scenario. Female preference in combination with FDS on morph juvenile can greatly accelerate RPS dynamics, explaining why common lizards exhibit rapid RPS dynamics and suggesting that RPS cycles may evolve under less restricted conditions.

Key words: Colour polymorphism; Context-dependent mate choice; Density-dependent selection; *Lacerta vivipara*; Theory of games; *Uta stansburiana*.

Evidencias Experimentales a favor de Selección Sexual Dependiente de Frecuencia y de Supervivencia de Juveniles Dependiente de Morfotipos como Inductores de Ciclos Rápidos de Piedra-papel-tijera de Morfotipos de Color en Machos

Resumen: La selección dependiente de frecuencia (SDF) sobre polimorfismos de color y las tácticas reproductivas asociadas pueden dar lugar a dinámicas complejas de piedra-papel-tijera (PPT), donde la frecuencia de tres morfotipos de color distintos ciclan alternándose la dominancia de cada estrategia en la población. Recientemente, se ha descrito la existencia de ciclos PPT rápidos en los morfotipos naranja, blanco y amarillo de los machos de lagartija de turbera, *Lacerta vivipara*. Sin embargo, la lagartija de turbera presenta ciertas características biológicas; alta longevidad y marcada estructura de edades, que se ha sugerido que alargan los ciclos PPT al impedir el recambio generacional y, por tanto, los cambios anuales en las frecuencias de morfotipos de color. Para reconciliar los rápidos ciclos PPT observados en la lagartija de turbera con las condiciones teóricas bajo las que se espera que los ciclos PPT evolucionen, se ha teorizado que las dinámicas PPT de la lagartija de turbera deberían ocurrir si **i.** las hembras eligen a los morfotipos como pareja de una forma dependiente del contexto y si **ii.** los morfotipos son reclutados desde juveniles de una forma dependiente de frecuencia. Testamos estas predicciones mediante seis poblaciones seminaturales de la lagartija de turbera, donde manipulamos la frecuencia de morfotipos de las cohortes de machos adultos y de machos de un año para reproducir dos fases distintas del ciclo PPT; tres poblaciones fueron hacia una frecuencia mayor de blancos y tres poblaciones hacia una frecuencia mayor de naranjas. Encontramos que el éxito de emparejamiento de los machos depende de su morfotipo y de la fase del ciclo PPT establecida, con los machos naranjas teniendo un mayor éxito en poblaciones que iban hacia una mayor frecuencia de blancos y los machos amarillos con un mayor éxito en poblaciones que iban hacia una mayor frecuencia de naranjas. Además, demostramos que los juveniles engendrados por los morfotipos con mayor éxito reproductor en cada una de las fases de PPT sobrevivieron mejor que los juveniles engendrados por el resto de los morfotipos en las poblaciones. Todos juntos, nuestros datos apoyan que los morfotipos de color en la lagartija de turbera están sujetos a selección sexual y natural dependiente de frecuencia. Dado que el éxito reproductor de los machos fue congruente con la futura frecuencia esperada de morfotipos (*i.e.*, con la frecuencia de morfotipos entre los machos de un año) y no con la frecuencia actual en adultos, nuestro estudio apoya que la selección sexual dependiente de frecuencia es debida a que las hembras eligen a sus parejas en función del contexto. Las hembras pueden sopesar la frecuencia de morfotipos en los juveniles de un año como una aproximación a la futura frecuencia de PPT que su progenie experimentará y pueden elegir como padres a aquellos morfotipos que mejoren la eficacia de la progenie en dicho futuro escenario. Las preferencias de las hembras en combinación con la SDF sobre los morfotipos en los juveniles puede acelerar enormemente las dinámicas de PPT, explicando por qué la lagartija de turbera presenta rápidas dinámicas de PPT y que los ciclos de PPT pueden evolucionar bajo condiciones menos restrictivas y, por tanto, ser mas comunes de lo previsto.

Introduction

Populations of a species can exhibit two or more genetically determined colour morphotypes that are too frequent to be the result of recurrent mutation (Huxley 1955). Fisher (1929) hypothesized that the presence of stable polymorphisms might result from a negative correlation between selective forces and genotypic frequencies of the population. This mechanism of selection, known as negative frequency-dependent selection (negative FDS), states that the fitness of a particular genotype varies with its frequency, increasing as the genotype becomes rarer in the population and decreasing as it becomes more frequent (Kojima 1971; Ayala and Campbell 1974; Heino et al. 1998). The ultimate consequence of negative FDS is that none of the alleles involved in the genetic determination of morphotypes spread to fixation, leading to the coexistence of morphotypes (Judson 1995). Despite Fisher (1929) himself recognized that FDS may "scarcely be other than exceptional" or, in words of Wright and Dobzhansky (1946), "an extreme hypothesis", increasing evidences avail the importance of negative FDS to explain the occurrence of different colour morphotypes within a population, especially in the context of interspecific interactions (*e.g.*, predation; Endler and Greenwood 1988; Olendorf et al. 2006; Fitzpatrick et al. 2009). However, despite theoretical framework has been extensively developed, less empirical evidences (particularly experimental) support the importance of FDS arising from intraspecific interactions in the maintenance of colour polymorphism (Sinervo et al. 2000; Gray and McKinnon 2007).

Colour polymorphisms are often associated with distinct alternative mating or life history strategies by being genetically correlated with morphological, physiological, behavioural, and life-history traits (Gross 1996; McKinnon and Pierotti 2010). When three alternative strategies coexist in interbreeding populations, FDS may maintain colour polymorphisms by sustaining complex rock-paper-scissors (RPS) dynamics (Wright 1968; Maynard Smith 1982; Nowak 2006; Sinervo and Calsbeek 2006). In a pure RPS dynamic, the strategy A dominates B, B dominates C, and C dominates A (Nowak 2006), thus, showing true RPS fitness intransitivity in which rare A but not rare C can invade common B populations, rare B but not rare A can invade common C populations, and rare C but not rare B can invade common A populations (Sinervo and Calsbeek 2006). Because no strategy is an evolutionary stable strategy (Maynard Smith 1982), fitness intransitivity triggers cyclical dynamics where colour morphs alternate as the common type in the population (Sinervo and Lively 1996). Although suggested to be common in nature, only a few biological RPS cycles have been described so far (Sinervo and Calsbeek 2006). The difficulty to manipulate frequencies of colour morphotypes at the population level hinder getting experimental evidence availing that FDS on alternative mating strategies sustains RPS dynamics and associated colour polymorphism (Bleay et al. 2007). However, it is the experimental manipulation of population frequencies of colour morphotypes and, hence, of frequencies of

alternative mating strategies, which may rigorously evidence covariation of fitness and frequency (Brockmann 2002; Shuster 2010).

In the Pyrenean populations of the common lizard (*Lacerta vivipara*), Sinervo et al. (2007) proposed that males show three colour morphs (orange, *o*, yellow, *y*, and white, *w*), which are akin to the orange, yellow, and blue male morphs described in the North American side-blotched lizard, *Uta stansburiana* (Sinervo and Lively 1996). Male colour morphs in the common lizard are based on a single putative locus with three alleles that produce six distinct phenotypes (Sinervo et al. 2007). Male common lizards exhibiting homogeneous orange (*oo*), white (*ww*), or yellow (*yy*) ventral colorations have been described as putative homozygotes and males showing mosaic-like colorations made up of two distinct colours (white-orange; *wo*, yellow-orange; *yo*, and white-yellow; *wy*) as putative heterozygotes (see fig. 1 in Sinervo et al. 2007). In natural common lizard populations, the frequency of male *o, w*, and *y* alleles varies describing a cyclical dynamic where high *y*, high *w*, and high *o* allele frequencies consecutively alternate within a period of 3 to 4 years (4 to 8 years at high elevation sites; Sinervo et al. 2007). As described in *Uta stansburiana* (Sinervo and Lively 1996), cycles of colour allele frequencies in *L. vivipara* have been suggested to result from RPS dynamics, where the cooperative *w* strategy invades the sneaky *y* strategy, the despotic *o* strategy invades the cooperative *w* strategy, which is in turn invaded by the sneaky *y* strategy (Sinervo et al. 2007). However, the rapid RPS dynamics found in the common lizard cannot be explained because of direct competition among adult male strategies as described in *U. stansburiana* (Sinervo et al. 2007). *Uta stansburiana* exhibit a short life span, which allows for rapid generational replacement and, hence, for effective morph frequency changes resulting from male controlling access to female harems in a RPS manner (Sinervo and Lively 1996). In contrast, common lizards are relatively long-lived and they are sexually active at the age of 1 to 2 years, which leads to a marked age structure with two or more distinct age classes (Heulin 1997; Massot et al. 2011). The occurrence of marked age classes is predicted to lengthen RPS dynamics because it favours the overlap of the allele frequencies of different generations as they are recruited into the adult cohort, which delays when a given colour allele becomes the most frequent in the population (Sinervo and Lively 1996; Zamudio and Sinervo 2003).

Theoretical models show that negative FDS on male colour polymorphism should involve younger cohorts as well as female context-dependent morph choice in order to sustain the rapid RPS cycles observed in the common lizard. Theory predicts that the strategies exhibited by adult males impair recruitment of juveniles as a function of juvenile morphotype and RPS phase (Sinervo et al. 2007). Models specifically predict that sneaky *y* juveniles survive better when despotic *o* adults are common, cooperative *w* juveniles survived better when sneaky *y* adults are common, and despotic *o* juveniles survived better when cooperative *w* adults are common (Fig. 1a; Sinervo et al. 2007). As a consequence of male limiting juvenile recruitment,

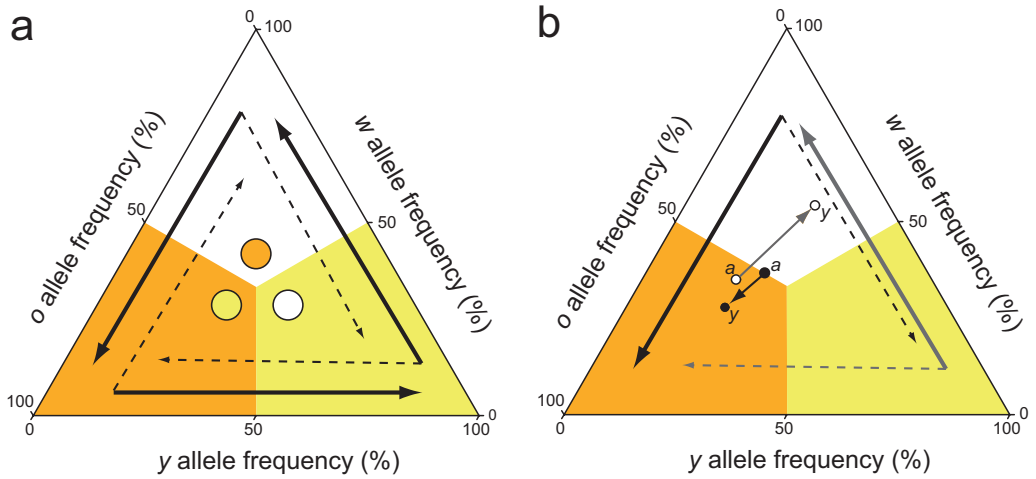

FIG. 1.—a. Ternary plot summarizing the expected pay-offs in the RPS cycle of *Lacerta vivipara*. Colourful areas represent the areas where *o*, *w*, and *y* alleles are the most frequent. Circles represent which allele is expected to confer a higher juvenile survival probability given the common allele in the population. Solid arrows represent the direction of the RPS cycle. Dashed arrows point to the male colour allele that is expected to confer a higher mating success in each RPS phase. **b.**Ternary plot of the experimental design for studying male colour morph cycles. Experimental biases of allele frequencies of yearling (y) and adult (a) cohorts in populations going to high *w* frequency (empty dots) and high *o* frequency (filled dots). Arrows connecting dots represents the vector of change of populations going to high *w* frequency (grey arrow) and of populations going to high *o* frequency (black arrow). Grey and black dashed lines indicate predicted mating success for populations going to high *w* frequency and high *o* frequency, respectively.

female context-dependent mate choice is predicted to evolve in order to enhance offspring recruitment (Sinervo et al. 2007). Thus, females are predicted to choose males whose morphotype inherited by juveniles has enhanced payoffs in the next generation (Alonzo and Sinervo 2001). Considering the above-stated effects of adult males on juvenile recruitment, females are predicted to mate with *y* males as *o* invades *w*, with *w* males as *y* invades *o*, and with *o* males as *w* invades *y* (Fig. 1a; Sinervo et al. 2007). Thus, females strategically enhance the recruitment of their progeny by anticipating the morphs frequency their offspring will experience. Mating success of adult male morphotypes is therefore predicted to depend on the expected future morphotype frequency (*i.e.*, in the vector of frequency change) instead of in the current frequency.

Here, we experimentally tested whether male mating success and juvenile survival are a function of colour morphs and RPS phase using six semi-natural populations of common lizards. We established three populations in the normal RPS direction by biasing the morphotype frequency of the adult (2+ years old) towards *o* alleles and the yearling cohort (1 year old) towards a higher *o* allele frequency (Fig. 1b). The vector of change of these populations was thus set as going towards high *o* frequency, given that *o* frequency is expected to increase as individuals in the yearling cohort are recruited. The remaining three popu-

lations were manipulated against the normal RPS direction. For this purpose, we biased the morphotype frequency of the adult cohort towards *o* alleles and biased the yearling cohort towards high *w* allele frequency. Thus, the vector of change of these populations was set as going to high *w* frequency but against the normal direction of the RPS cycle (*i.e.*, from high *o* to high *w* instead of from high *y* to high *w*; Fig. 1). By manipulating allele frequency of the yearling cohort but not the allele frequency of the adult cohort, we were able to test if morph fitness depends upon the expected next frequency (*i.e.*, in the vector of change) or, instead, if it depends upon current adult morph frequency, as observed in *U. stansburiana* (Sinervo and Lively 1996). According to RPS predictions based on female mate-choice (Fig. 1a), we expect that, in populations going to high *o* frequency, males having more *y* alleles have a higher mating success, given that the recruitment of juveniles inheriting *y* alleles is predicted to be favoured as *o* becomes the dominant allele in the population (Fig. 1b). In populations going to high *w* frequency, we expect that *o* males have a higher mating success, given that the recruitment of juveniles inheriting *o* alleles is predicted to be favoured as *w* becomes the most frequent allele. On the contrary, if morph fitness does not depend upon the vector of change but on the current adult morph frequency, we expect no differences between treatments in morph mating success given that in both treatments males having more *w* alleles should exhibit a higher mating success according to RPS predictions (Fig. 1). Alternatively, if morph fitness depends upon competition among adult males as described for *U. stansburiana*, we would also expect no differences between treatments given that, in such circumstances, males having more *y* alleles should exhibit a higher mating success in all populations (rare *y* sneakers successfully cuckold females when *o* males are common; Sinervo and Lively 1996). According to the RPS predictions for juvenile recruitment, we expect that, in populations going to high *o* frequency, juveniles sired by males with increasing *y* alleles (*i.e.*, those with maximized fitness in the context of the RPS dynamic; Fig. 1a) survive better, given that *y* juveniles have higher payoffs when *o* alleles are common in the adult cohort. Similarly, in populations going to high *w* frequency, we expect that juveniles sired by males with increasing *o* alleles (*i.e.*, those with predicted maximized fitness) survive better, given that *o* juveniles have higher payoffs when *w* alleles are common in the adult cohort.

Materials and Methods

Experimental manipulation of morphotype frequencies

In September 2008, we established six semi-natural populations at the research station 'El Boalar' (42°33'N, 0°37'W, 700 m a.s.l.) of the Instituto Pirenaico de Ecología, Jaca, Spain. Populations (100 m^2) were enclosed using galvanized metal walls (1 m above ground and 1 m below ground) to prevent lizards from escaping. Nets hindered predators from entering. Each enclosure contained a patch of planted natural grassland, two water ponds, logs and four piles of stones, which provided lizards with food, water, shelter, and

basking sites. Twice a day, four irrigation sprinklers sprayed the surface of each enclosure, maintaining high humidity conditions during the entire day.

In each population, we released 12-13 adults (7 females and 5 to 6 adult males; more than 2 years old, ≥ 51 mm snout-to-vent length, SVL), 6-7 yearlings (3 females and 3 to 4 males; 1 year old, 34 ≥ SVL ≤ 51 mm), and 6 juveniles (3 females and 3 males; born in 2008, 24 ≥ SVL ≤ 30 mm; Fitze and Le Galliard 2008). Males were scored according to their morphotype (Sinervo et al. 2007). We scored male colour morph using two colour scales: *o* scale: 0, 1, or 2 *o* alleles (0 = *ww*, *wy*, 1 = *yo*, *wo*, 2 = *oo*) and *y* scale: 0 or 1 *y* alleles (0 = *ww*, *oo*, *wo*, 1 = *wy*, *yo*; in 2008 no *yy* males could be capture in the field). In three populations, we biased the yearling male colour morph frequency towards orange morphotypes (populations going to high *o* frequency) and in the remaining three populations towards white morphotypes (populations going to high *w* frequency; *Pearsons'* χ^2 = 5.85, d.f. = 1, P = 0.016; Fig. 1b). Adult morphotypes frequency was held constant in all populations (*Pearsons'* χ^2 = 0.47, d.f. = 1, P = 0.491). The established frequency bias persisted in 2009 mating season (yearling cohort: *Pearsons'* χ^2 = 5.24, d.f. = 1, P = 0.022, adult cohort: *Pearsons'* χ^2 = 0.62, d.f. = 1, P = 0.43). Established morphotype frequencies were within the natural range of morph frequencies observed in natural populations (observed *w* allele frequency: mean ± SE = 0.35 ± 0.04, range = 0-0.75, observed *o* allele frequency: 0.40 ± 0.04, 0.13-0.67, observed *y* allele frequency: 0.25 ± 0.02, 0.06-0.40, statistics from 8 populations measured in two or three consecutive years, mean N = 33.3 males per population and year).

Juvenile rearing and survival

Once the mating season ended (end of May 2009), we captured all lizards and measured their SVL and body mass (to the nearest 1 mm and 1 mg, respectively). Gravid females were kept in individual *terraria* equipped with a shelter, a water pond, and peat soil as substrate. Water was provided *ad libitum* and females were fed every three days with different prey items (larvae of *Galleria mellonella, Acheta domestica,* and *Lumbricus terrestris*). Once a week, we floured *larvae* of *G. mellonella* with standard reptile supplements of calcium (Microcalcium TerraVit, JBL, Neuhofen, Germany) and vitamins (Nekton-rep, Nekton, Pfrozheim, Germany). A 40 W bulb illuminated the *terraria* and provided heat following a 10-h light:14-h dark photoperiod and a UV lamp provided UVB and UVA for one hour and half to assure calcium metabolism. Every morning and night, *terraria* were inspected for laid clutches. Clutches were inspected against a light to determine the presence of the germinal disk and, hence, to determine whether eggs were fertile or sterile (Kohler 2005). Clutches were weighed (to the nearest 1 mg), placed into a small recipient filled with fine river soil saturated with water, and incubated at 21 °C during day (from 09:00 h to 21:00 h) and 19 °C during night (Heulin 1997). Immediately after hatch, we measured SVL and body mass of all hatchlings. Sex determination of hatchlings was done by counting the number of ventral scales as described by Lecomte et al. (1992). All ju-

veniles were released within 24 h after hatching. To measure juvenile survival, we recaptured all juveniles at the end of September 2009 (pre-hibernation survival).

Paternity assessment

We collected a tissue sample from the tail of every lizard (including juveniles hatchling in 2009) and stored it in 70% ethanol. Unhatched eggs were entirely preserved in 70% ethanol. DNA was extracted using BioSprint 96 DNA Blood Kit (Qiagen, Hilden, Germany). Females laid a total of 140 eggs, 27 of which lacked a germinal disk and were considered sterile. Ninety-eight juveniles hatched from eggs classified as fertile and no juvenile hatched from sterile eggs. DNA from all hatchlings and of 9 of 15 unhatched fertile eggs was successfully extracted. DNA of 6 unhatched (5.3 % of fertile eggs) and fertilized eggs was degraded and paternity could not be attributed. Methods of DNA extraction, polymerase chain reaction, and allele size determination are described elsewhere (Laloi et al. 2004). For paternity assessment, we used four to six polymorphic microsatellite DNA loci (Lv-3-19, Lv-4-72, Lv-4-alpha, Lv-2-145, Lv-4-X, Lv-4-115; Boudjemadi et al. 1999). Paternity assessment was done for each population separately using Cervus 3.0. (Marshall et al. 1998). Given that the genotype of mothers and of all potential father was known, Cervus 3.0. was simply used to facilitate the attribution of the genetic father. Offspring was successfully attributed to a single father.

Statistical analyses

We used generalized linear mixed models with a quasipoisson distribution to investigate the effects of RPS phase and *o* and *y* scale on the number of male mate partners (*i.e.*, number of females with which a male sired eggs, hereafter referred to as mating success) and the total number of eggs sired per male. Full models include RPS phase, *o* and *y* scales (and their interactions), and male SVL as fixed factors and population nested within RPS phase as a random factor. We thereafter investigated whether, as predicted by RPS dynamics, juvenile survival was dependent upon sire morphotype and RPS phase. Using generalized linear mixed models with a binomial distribution we tested for differences in prehibernation (from hatchling to September'09) survival of juveniles born in 2009. Because no *wy* male sired offspring in populations going to high *w* frequency (see results), we could not test for an interaction between colour scales and RPS phase on juvenile survival. Instead, we tested whether survival of juveniles sired by male morphotype exhibiting a higher mating success in each RPS phase (*i.e.*, mates with the highest-payoff sire morphotype; Fig. 1a) differed from survival of juveniles sired by the remaining male morphotypes in the populations. Full models included RPS phase, sire mating success (high *vs.* low), juvenile sex and their interactions as fixed effects and population nested within RPS phase and juvenile mother as random factors. As covariates, we included hatching date and juvenile SVL at hatching. We tested whether sex, hatching date, SVL at hatching, and body mass at hatching of juveniles were dependent on RPS phase and sire morphotype, using mixed-model ANCOVAS with RPS phase, sire mating success and their interaction as fixed fac-

tors and population nested within RPS phase and juvenile mother as random factors. All statistical analyses were done using the package lme4 implemented in R 2.10.1 (R Development Core Team 2008). The best model in each analysis was obtained by backward elimination ($P > 0.05$). Multiple testing was accounted for following Hochberg procedures (Benjamini and Hochberg 1995).

Results

Male mating success (*i.e.*, the number of females with which a male sired offspring), significantly depended on the interaction between RPS phase and the number of putative *y* and *o* alleles of males (RPS phase × *o* scale: $\chi^2 = 13.58$, d.f. = 1, $P < 0.001$, RPS phase × *y* scale: $\chi^2 = 7.90$, d.f. = 1, $P = 0.005$, RPS phase × *o* scale × *y* scale: $\chi^2 = 5.09$, d.f. = 1, $P = 0.024$). In populations going to high *o* frequency, male mating success increased with increasing *y* and decreasing *o* scale (Fig. 2a) while males with less *y* alleles and more *o* alleles had a higher mating success in populations going to high *w* frequency (Fig. 2b). In populations going to high *o* frequency, *wy* males had the highest mating success (mean ± SE: 2.5 ± 0.89 females), and *oo* males had thde lowest mating success (mean ± SE: 0.42 ± 0.24 females). In populations going to high *w* frequency, *oo* males mated with more females (mean ± SE: 3.67 ± 1.45 females) and *wy* males showed the lowest mating success (none of the *wy* males sired eggs in populations going to high *w* frequency). Male mating success was also positively related with SVL (estimate ± SE: 0.11 ± 0.01; $\chi^2 = 16.62$, d.f. = 1, $P < 0.001$), indicating that larger

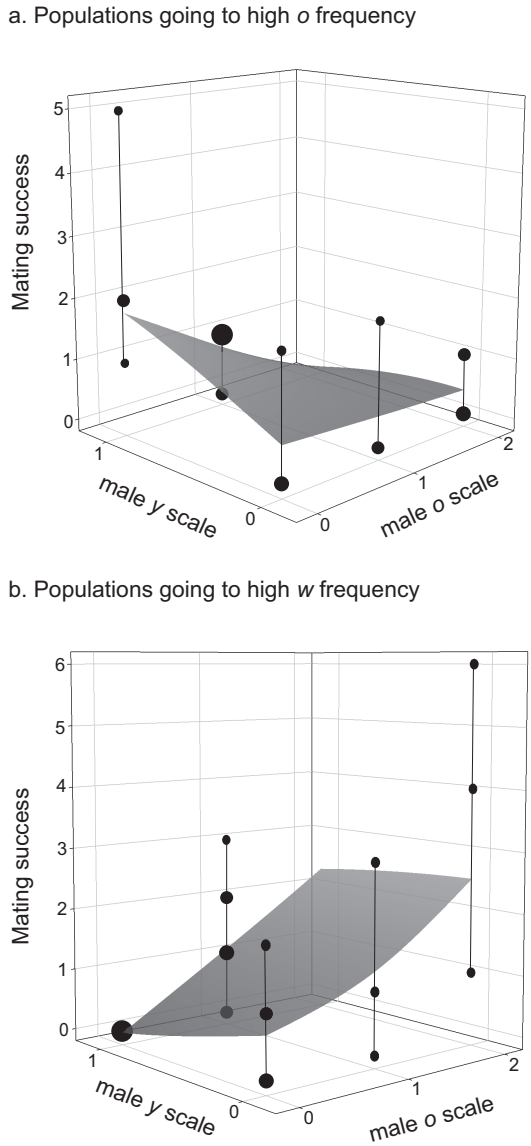

Fig. 2.—Predicted surface for morph mating success (number of females with which a male morphotype sired offspring) as a function of male *o* and *y* scales for **a.** populations going to high *o* frequency and for **b.** populations going to high *w* frequency. Dots represent observed values and dot size is proportional to the number of observed values.

males mate with more females. The mean number of eggs sired per female did not significantly depend on male colouration (*o*

scale: $\chi^2 = 0.06$, d.f. = 1, $P = 0.89$; *y* scale: $\chi^2 = 0.41$, d.f. = 1, $P = 0.52$; *o* scale × *y* scale: $\chi^2 = 0.01$, d.f. = 1, $P = 0.92$), RPS phase ($\chi^2 = 0.02$, d.f. = 1, $P = 0.89$), or their interaction (RPS phase × *o* scale: $\chi^2 = 0.29$, d.f. = 1, $P = 0.59$, RPS phase × *y* scale: $\chi^2 = 1.63$, d.f. = 1, $P = 0.20$). Because no *wy* male sired offspring in populations going to high *w* frequency, the interaction between RPS phase, *o* scale, and *y* scale could not be analyzed. The mean number of eggs a male sired per female did not depend on male SVL ($\chi^2 = 0.46$, d.f. = 1, $P = 0.83$).

Offspring traits (hatching date, sex, SVL, body mass) did not significantly differ between offspring sired by male morphotypes that exhibited a higher mating success (*i.e.*, juveniles sired by *wy* males in populations going to high *o* frequency and juveniles sired by *oo* males in populations going to high *w* frequency) and those sired by males of the remaining morphotypes (Table 1). However, offspring sired by male morphotypes that exhibited a higher mating success showed higher prehibernation survival than offspring sired by the remaining morphotypes ($\chi^2 = 6.50$, d.f. = 1, $P = 0.011$; Fig. 3). This effect was independent of RPS phase and juvenile sex as revealed by non-significant interaction between RPS phase and sire success ($\chi^2 = 0.36$, d.f. = 1, $P = 0.55$), between RPS phase and juvenile sex ($\chi^2 = 0.53$, d.f. = 1, $P = 0.465$), and between RPS phase, juvenile sex and sire success ($\chi^2 < 0.01$, d.f. = 1, $P = 0.98$). Prehibernation sur-

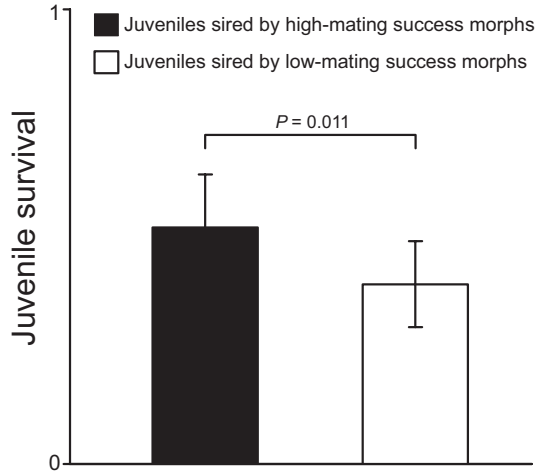

FIG. 3.—Mean (± S.E.) prehibernation (from hatching to September'09) survival probability of juveniles sired by male morphotypes with higher mating success (wy males in populations going to high o allele frequency and oo males in populations going to high w allele frequency; black bar) and of juveniles sired by the remaining morphotypes (white bar).

TABLE 1.—Results from mixed model ANCOVAS of RPS phase, sire mating success (high: *wy* and *oo* males in populations going to high *o* and high *w* frequency, respectively, *vs.* low: remaining morphs in the population) and their interaction in juvenile sex, hatching date, and SVL and body mass at hatch.

	RPS phase		Sire mating success		Interaction	
Variable	Test statistic	*P*	Test statistic	*P*	Test statistic	*P*
Sex	$\chi^2 = 1.43$	0.23	$\chi^2 = 0.48$	0.49	$\chi^2 = 0.76$	0.38
Hatching date	$F_{1,69} = 0.32$	0.57	$F_{1,70} = 1.11$	0.30	$F_{1,65} = 0.45$	0.50
SVL	$F_{1,67} = 0.01$	0.92	$F_{1,69} = 1.24$	0.27	$F_{1,65} = 0.38$	0.54
Body mass	$F_{1,62} = 0.21$	0.65	$F_{1,64} = 0.69$	0.41	$F_{1,61} = 0.87$	0.35

vival depended on hatching date (χ^2 = 24.76, d.f. = 1, P < 0.001) and sex (χ^2 = 4.31, d.f. = 1, P = 0.038). Juveniles born later in the year survived worse than earlier hatched juveniles (estimate ± SE: –0.07 ± 0.02) and female juveniles survived better than male juveniles (estimate ± SE: –1.28 ± 0.6).

Discussion

Negative FDS on colour polymorphisms and associated mating strategies may generate complex RPS dynamics (Sinervo and Calsbeek 2006; Sinervo and Calsbeek 2010). Although suggested to be common at the species and ecosystem levels (review in Sinervo and Calsbeek 2006), RPS dynamics has been described only in a few species (Sinervo and Lively 1996; Kirkup and Riley 2004). In the Pyrenean populations of the common lizard, male colour morph frequencies exhibit rapid RPS cycles although common lizards exhibit certain biological features (long life span and the occurrence of two or more age classes) that are expected to hinder frequency changes and, hence, to lengthen RPS cycles (Sinervo et al. 2007). Theory suggests that the yet rapid RPS cycles observed in the common lizard may occur if **i.** juvenile morphotypes are recruited as a function of the dominant morphotype in the adult cohort and **ii.** if female choose sire morphotypes in order to enhance the recruitment of their offspring (Sinervo et al. 2007). Here, we provide experimental evidences for both suggested mechanisms of FDS selection.

We demonstrated that male mating success depends on male colour morphotype and the phase of the RPS cycle. We showed that males with more *y* and less *o* alleles had higher mating success in populations going to high *o* frequency whereas males having more *o* and less *y* alleles had the highest mating success in populations going to high *w* frequency. The observed mating bias could be the result of different sexual selection pressures. Mating bias may occur if male morphs control access to females in a frequency-dependent manner or if females choose male morphs as a function of morph frequency (Kokko et al. 2003). If the observed mating bias would have resulted from adult male morphs controlling access to females, we should have observed no differences between treatments in morph mating success, given that similar adult morph frequency was established in all populations (Fig. 1b). Thus, if, as described for *U. stansburiana*, the observed mating bias was mainly the consequence of male morphotypes competing for access to females, we should have observed rare sneaky *y* males to have the highest fitness in all populations because they have an advantage in cuckolding females when despotic *o* males dominate in the adult cohort (Sinervo and Lively 1996; Zamudio and Sinervo 2000; Sinervo et al. 2007).

Contrary, male fitness depended on colour morph as well as in the vector of allele frequency change. As predicted, males with more *y* alleles and less *o* alleles mated with more females in populations moving towards a higher *o* frequency whereas males having more *o* alleles and less *y* alleles mated with more females in populations moving to high *w* frequency (Fig. 1b; Sinervo et al. 2007). Therefore, morph fitness varied according to the dominant allele frequency in the yearling cohort and, thus, according to the expected fu-

ture frequency. This finding supports that context-dependent female mate-choice rather than male-male competition accounted for the observed mating bias. Females might have chosen distinct male morphs in the face of different future RPS scenarios and, hence, according to the common allele frequency that offspring will encounter (Alonzo and Sinervo 2001; Sinervo et al. 2007). Context-dependent mate choice depends on the occurrence of reliable cues to predict the quality of the environment that offspring will experience (Qvarnström 2001). Given that RPS cycles in the common lizard may extend for up to 8 years and, hence, an allele may be the most frequent for more than one year (Sinervo et al. 2007), the current adult frequency may not constitute a reliable cue of frequency changes. On the contrary, allele frequency in the yearling cohort is expected to be a more accurate proxy of frequency changes, given that it reflects which will be the most frequent allele frequency in the next generation. Female mate choice may be plastic because direct and indirect benefits of mating are generally context-dependent (Qvarnström 2001; Kokko et al. 2003; Welch 2003; Fitze et al. 2010). Particularly, female context-dependent mate choice is predicted to occur in the common lizard in order to enhance offspring survival (Alonzo and Sinervo 2001; Sinervo et al. 2007). Our results indicate that progeny survival indeed depended on sire morphotype. Juveniles sired by most preferred morphotypes exhibited higher survival than juveniles sired by the other morphotypes present in the populations, supporting that context-dependent female mate choice may be selectively advantageous through indirect benefits (Kokko et al. 2003).

We may discard that the effects on juvenile survival resulted from maternal effects (Sorci and Clobert 1997; Meylan and Clobert 2004) given that we found no differences among juveniles supporting that females invested more in juveniles sired by the most preferred morphotypes. Differences in juvenile survival more likely resulted because of the different social environments experienced by juveniles (Sinervo et al. 2007). By the time juveniles hatched and were released into their respective populations, yearling cohort established in 2008 was successfully integrated in the adult cohort, leading to common *w* adult populations and to common *o* adult populations. In common *w* adult populations, juveniles sired by males with more *o* alleles and less *y* alleles (*i.e.*, the preferred males in these populations, *oo* males) may have an advantage for survival because *o* alleles confer a higher competing ability against common *w* adults (Sinervo et al. 2007). In contrast, in common *o* adult populations, juveniles sired with less *o* alleles and more *y* may have an advantage for survival because sneaky *y* juveniles may avoid engaging into antagonistic interactions with common *o* adults (Sinervo et al. 2007). Because juveniles exhibit no coloration, we cannot attribute juvenile morphotype with a higher precision and it is likely that some of the juveniles sired by non-preferred morphotypes also inherited a high-payoff genotype and that some of the juveniles sired by preferred morphotypes inherited a low-payoff genotype. Nevertheless, the fact that we observed the expected differences in juvenile survival indicates that selec-

tion imposed by adult cohort on juvenile survival may be stronger than here detected. Inter-cohort competition has been previously suggested in the common lizard (Lecomte et al. 1994). Adult males are known to display aggressive behaviours towards smaller individuals (Léna and de Fraipont 1998) and, although it may not be the rule in natural populations, adult males has been observed to prey on juveniles in captivity (Léna and de Fraipont 1998; L. M. San-Jose, personal observation). Juvenile survival has been demonstrated to decrease under conditions that may induce higher levels of intraspecific competition like in high adult density (Massot et al. 1992) or when juveniles colonize populations with a low frequency of related individuals (Cote et al. 2007). In order to minimize potential competition, juvenile common lizards disperse in a density-dependent manner (Massot et al. 1992; Galliard et al. 2003). Dispersal probability is usually associated with juvenile phenotype (Léna et al. 1998; Meylan and Clobert 2004; Cote and Clobert 2007b) and depends on the assessment of particular environmental cues (Aragon 2006; Aragon et al. 2006; Cote and Clobert 2007a). In the light of our findings, we expect juvenile dispersal to also depend on RPS dynamics and that juveniles may disperse according to their morphotype and the dominant strategy in their natal populations (Sinervo and Clobert 2003).

Our findings provide experimental support for the complex social interactions that may lead to the maintenance of colour polymorphism. Negative FDS on mating success as well as on survival of colour morphotypes may avoid the fixation of colour alleles maintaining colour variation in male common lizards. RPS cycles has been previously suggested to evolve in species if sexual selective process allows for high mating bias, if females reproduce several times, which enhances mating bias, and, finally, if life span is short, which avoids the establishment of a marked age structure (Zamudio and Sinervo 2003; Sinervo et al. 2007). The here-observed different selective forces on colour morphotypes; *i.e.*, mating bias through context-dependent selection and frequency-dependent recruitment of juvenile morphotypes, help to understand the existence of rapid RPS cycles in the face of a well-established age structure (Sinervo et al. 2007). The combination of these distinct selective pressures allows substantial changes in colour allele frequencies among parental and filial generations, minimizing the mixture of the allele frequencies of older and younger cohorts and, thus, the length of RPS cycles. The fact that RPS cycles may indeed evolve in species with a marked age structure if they exhibit inter-cohort regulation, indicates that the conditions required for the evolution of RPS dynamics may be less restrictive than previously thought, which support that RPS cycles may be more common in nature (Sinervo and Calsbeek 2006).

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Chapter VII

Resumen de Resultados y Discusión General

Los resultados obtenidos en este trabajo permiten concluir que los pigmentos responsables de la coloración anaranjada del vientre de la lagartija de turbera, *Lacerta vivipara*, son de tipo carotenoide (capítulos II y III). Otros pigmentos, las pteridinas, fueron descartados como pigmentos responsables de la coloración anaranjada de *L. vivipara* (capítulo II). Este hecho contrasta con anteriores estudios en otras especies de lagartijas donde se observó que las pteridinas determinan en gran medida o incluso de forma exclusiva la coloración (Olsson et al. 2008; Steffen y McGraw 2009; Weiss et al. 2011). Igualmente, la ausencia de pteridinas en el integumento de *L. vivipara* contrasta con otros estudios realizados en peces donde se observó que, según se hacen más escasos los carotenoides en la dieta, los individuos sintetizan e incorporan más pteridinas en el integumento para producir ornamentos más rojos y llamativos (Grether et al. 2005).

Se encontraron diferencias sustanciales en la composición de carotenoides de la piel de individuos del clado ovovivíparo (capítulo II) y ovíparo (capítulo III) de Europa occidental. Aunque luteína y zeaxantina, dos xantófilas muy comunes en los tejidos de otras especies de lagartija así como de aves (Costantini et al. 2005; Hill y McGraw 2006; Steffen et al. 2010), fueron comunes a ambos clados, otros carotenoides fueron encontrados de forma dispar en uno u otro clado (astaxantina y cantaxantina en el clado ovovivíparo y β-caroteno en el clado ovíparo; capítulos II y III). El presente estudio tampoco presenta una composición semejante a la presentada por Czeczuga (1980), quien encontró una mayor diversidad de carotenoides a la aquí encontrada tanto en piel como en hígado (capítulos II y III). Los estudios de Czeczuga (1980) se realizaron en lagartijas del clado ovovivíparo de Europa occidental, lo que apoya que las diferencias observadas entre los capítulos II y III del presente estudio es más probable que indiquen variación interpoblacional que diferencias genéticas entre clados (Negro et al. 2000; Tella et al. 2004). Siendo así, la variabilidad en los tipos de carotenoides presentes en la piel de *L. vivipara* podría tener un origen ambiental, pudiendo estar determinada por la disponibilidad en la dieta de unos u otros tipos de carotenoides (Negro et al. 2000). Este hecho contrastaría, tal y como se discute líneas más abajo, con la aparentemente nula determinación ambiental de la concentración de carotenoides en la piel (capítulos II, IV, y V) y merece, por tanto, especial atención en futuros estudios.

El presente estudio arroja resultados contradictorios en cuanto a qué parámetros del color están directamente relacionados con los carotenoides en la piel. En el capítulo II se observó que la concentración de carotenoides se relaciona con el tono ('hue') mientras que en el capítulo V se observó que la concentración de carotenoides se relaciona con la reflectancia medida en la región donde los carotenoides absorben luz (*i.e.*, entre 400-500 nm de longitud de onda) pero no con el tono. La acumulación de concentraciones mayores de carotenoides en el integumento generalmente conlleva cambios en la reflectancia entre 400-500 nm mientras que la adición de distintos tipos de carotenoides da lugar a cambios en el tono (Andersson y Prager 2006; ver también capítulo I). Por lo tanto, los distintos resultados obtenidos en los capítulos II y V quizás reflejen distintos patrones de variación entre individuos en la acumulación de carotenoides del integumento. Así, en el capítulo II, la variación de color entre individuos podría estar determinada principal-

mente por la variación en la predominancia de unos carotenoides sobre otros mientras que la variación de color entre los individuos medidos en el capítulo V vendría motivada principalmente por cambios en la concentración de carotenoides (capítulo V).

Ya los primeros estudios en ornamentos basados en carotenoides (Endler 1980; Endler 1983) señalaban la capacidad de éstos para reflejar las condiciones ambientales debido a que los carotenoides están sujetos a diversas limitaciones que finalmente impiden que los individuos inviertan carotenoides de forma ilimitada en sus ornamentos (Badyaev y Hill 2000). La principal limitación surge del hecho de que los animales no pueden sintetizar los carotenoides *de novo*, lo que les obliga a obtenerlos con la dieta (Goodwin 1986). En consecuencia, la expresión del color se convierte en una función de la cantidad de carotenoides obtenidos con la dieta, como se ha demostrado de forma experimental en varias especies de aves y peces (ver Tschirren et al. 2003 y Kodric-Brown 1989 para un ejemplo en aves y peces, respectivamente). Sin embargo, a lo largo del presente estudio no se observó este efecto y de forma repetida se encontró que la expresión del color en *L. vivipara* es independiente de suplementar la dieta con carotenoides (capítulos II, IV, y V). Se demostró que suplementar con carotenoides induce un incremento de los niveles circulantes de carotenoides (capítulos II, IV, y V) así como de la concentración de carotenoides en el hígado (capítulo V), principal órgano de reserva de carotenoides (capítulo III). Así mismo, se observó que la cantidad de carotenoides en los tejidos de las lagartijas depende de otros factores como la ingesta de lípidos, la cual afecta de forma negativa a la cantidad de carotenoides en sangre (capítulo IV), y como la síntesis de vitamina A, la cual pudo ser la causa de que suplementar la dieta con β-caroteno no se reflejase en el incremento de este carotenoide en los distintos tejidos analizados (capítulo V). Estos resultados apoyan que, tal y como se ha observado anteriormente en peces y aves (Grether et al. 1999; McGraw y Ardia 2003), la cantidad de carotenoides acumulada en los tejidos de *L. vivipara* depende de distintos factores ambientales.

Sin embargo, los cambios inducidos en la concentración tisular de carotenoides por los distintos suplementos realizados no afectaron a ninguno de los parámetros de color medidos (capítulos II, IV, y V) ni tan siquiera cuando suplementar la dieta con carotenoides aumentó de forma significativa los niveles de carotenoides (luteína y zeaxantina) en la piel (capítulo V). Estos hechos corroboran que la cantidad de carotenoides que *L. vivipara* utiliza para la coloración ventral está sujeta a la disponibilidad de carotenoides o a los factores ambientales que determinen dicha disponibilidad y apuntan hacia un mayor control genético. La relación entre la cantidad de carotenoides de la piel así como de la reflectancia debida a éstos con los morfotipos de color de los machos de *L. vivipara* (capítulo V) apoya esta hipótesis. Se observó que los morfotipos más coloreados (*i.e.*, aquellos con un mayor número de alelos naranjas y amarillos) muestran una mayor concentración de carotenoides en la piel y, consecuentemente, una menor reflectancia en la región espectral dependiente de los carotenoides (capítulo V). Los polimorfismos de color tienen por lo general una estricta determinación genética (Sinervo et al. 2001; Sinervo et al. 2006; Vercken et al. 2007). Dicho control genético es necesario ya que, de lo contrario, una expresión de los morfotipos en función del ambiente daría lugar a un desajuste de las estrategias representadas por los morfotipos de color (capítulo VI; Sinervo et al. 2007).

Por lo tanto, el color basado en carotenoides indica distintas estrategias en los machos y sirve como señal indicadora en las interacciones sociales. Los morfotipos de color de *L. vivipara* determinan de forma sustancial la eficacia biológica de los machos (capítulo VI; Sinervo et al. 2007). Se encontró que el color está bajo selección sexual y natural dependiente de frecuencia (capítulo VI). En concreto, se observó que la supervivencia de los juveniles es una función del morfotipo del padre en interacción con la frecuencia de morfotipos de la población y que el éxito reproductor en los machos depende de su propio morfotipo en interacción con la frecuencia de morfotipos que su progenie experimentará en la siguiente generación (capítulo VI). Estos mecanismos de selección dependiente de frecuencia explican la aparición de ciclos de piedra-papel-tijera donde la frecuencia de cada morfotipo de color varía a lo largo del tiempo, manteniendo la variabilidad de distintas formas de color en las poblaciones naturales de *L. vivipara* (capítulo VI; Sinervo et al. 2007).

Los datos presentados aquí cuestionan la generalidad de uno de los principales paradigmas por el cual se ha demostrado que se rigen las coloraciones basadas en carotenoides de peces y aves, ya que en contra de lo observado en estos grupos de animales la cantidad de carotenoides que *L. vivipara* usa para el color no muestra plasticidad ambiental y, por lo tanto, el color no constituye una señal de la condición de los individuos. La cuestión que surge a colación de estos resultados es, ¿por qué *L. vivipara* presenta una menor plasticidad en relación a los carotenoides de la piel si, como ocurre en aves y peces, tampoco puede sintetizar carotenoides por sí misma? El hecho de que la acumulación de carotenoides en el integumento pueda estar supeditada a un mayor control genético no permite responder a esta pregunta, ya que dicha expresión genética no hace a los individuos independientes de la disponibilidad de carotenoides en el medio. Los resultados aquí presentados dan lugar a dos posibles escenarios para responder a la pregunta planteada. En un primer escenario, la ausencia de plasticidad fenotípica observada indicaría que *L. vivipara* no está limitada en la cantidad de carotenoides que pueden obtener del medio. Así los individuos obtendrían suficientes carotenoides con la dieta para hacer efectiva la determinación genética del color y expresar el color de forma congruente con su genotipo. En un segundo escenario donde los carotenoides sí que fuesen escasos en el medio, sería esperable que la expresión de los distintos morfotipos basados en carotenoides estuviese sujeta a distintos patrones de utilización de carotenoides entre su función ornamental y sus funciones de mantenimiento (función antioxidante e inmunoestimulante) en función del morfotipo. Así, los morfotipos que expresen coloraciones basadas en más carotenoides tendrán que hacerlo a expensas de tener una menor disponibilidad de carotenoides para funciones de mantenimiento. Aunque el presente estudio no permite discernir entre ambos escenarios, el hecho de que los morfotipos con más alelos naranjas y amarillos presentasen menores concentraciones de carotenoides en los tejidos de reserva que los morfotipos con más alelos blancos (capítulo V) es congruente con este último escenario. Igualmente, los morfotipos de color representan estrategias alternativas que generalmente incluyen diferencias en la respuesta inmunitaria o en la carga parasitaria (Dijkstra et al. 2007; Huyghe et al. 2010), lo que podría estar relacionado con un posible uso diferencial de los carotenoides por parte de los morfotipos.

A pesar de que los carotenoides acumulados en la piel y la parte espectral de la que éstos son responsables parece estar bajo control genético, la coloración ventral de *L. vivipara* mostró variación bajo condiciones ambientales cambiantes. Así, se observó que el color varía en función de la concentración de corticosterona (capítulos II y V; ver también Cote et al. 2010), en función de los lípidos de la dieta y su efecto sobre los niveles circulantes de vitamina E (capítulo IV), y en función de la ingesta de β-caroteno y, quizás, sobre el efecto de éste sobre los niveles de vitamina A (capítulo V). Como se mostró en el capítulo V, la plasticidad ambiental de la coloración ventral de *L. vivipara* tiene su origen en cambios en las propiedades reflectantes de los iridióforos, los cuales controlan la reflectancia de la región lejana del espectro visible dando lugar a cambios cromáticos en la coloración de *L. vivipara*. Probablemente, los cambios observados en otros capítulos a consecuencia de la corticosterona (capítulo II) y de los efectos de la ingesta de lípidos sobre la vitamina E (capítulo IV) así como en otros estudios en esta especie (Meylan et al. 2007; Cote et al. 2008; Cote et al. 2010) también fuesen el resultado de cambios en la reflectancia debida a iridióforos. Qué mecanismos relacionan los iridióforos con la condición es un tema aún inexplorado. Dichos mecanismos probablemente estén presentes en otras especies de lagartijas así como en otros reptiles en general, en peces y en anfibios, ya que todos ellos presentan iridióforos en su integumento (Grether et al. 2004). Desentrañar dichos mecanismos constituye el primer paso para averiguar cómo los iridióforos dan lugar a coloraciones que honestamente indiquen la condición.

Sorprendentemente, el presente estudio indica que los iridióforos podrían estar asociados con otros factores con los que también se ha observado que están relacionados los carotenoides. Por ejemplo, se ha observado que los carotenoides también pueden reflejar los niveles de corticosterona (Loiseau et al. 2008) o estar sujetos a variaciones en los niveles de vitamina E (Pike et al. 2007; Pérez et al. 2008). De igual forma, se ha observado que las coloraciones basadas en melanina pueden variar en relación a estos mismos factores (corticosterona; Roulin et al. 2008, y antioxidantes; Galvan y Alonso-Alvarez 2008) al igual que las coloraciones basadas en pteridinas, que también parecen asociarse a los niveles de antioxidantes (Weiss et al. 2006; Weiss et al. 2011). En conjunto, estos trabajos y el presente estudio evidencian que, de forma general, existe un marcado paralelismo en cuanto a qué componentes de la condición de los animales son señalizados por los distintos tipos de componentes que determinan el color en animales.

El hecho de que otros compuestos que no sean los carotenoides expliquen la variación cromática, es decir, aquella generalmente asociada a los carotenoides, muestra que los ornamentos basados en carotenoides pueden ser en realidad ornamentos basados en varios componentes y con varias dimensiones de complejidad. Este hecho conlleva importantes consideraciones metodológicas, ya que como se mostró en el capítulo V, el efecto de los iridióforos puede ser erróneamente atribuido a cambios en los carotenoides del integumento. Así, por ejemplo, si se consideran los efectos de la ingesta de β-caroteno el cual se asoció con coloraciones más saturadas y anaranjadas (capítulo V) reproduciendo los efectos previamente observados en aves y peces (*e.g.*, Andersson y Prager 2006), se podría concluir que los efectos fueron directamente debidos a que los animales, al tener una mayor disponibilidad de β-caroteno, pudieron invertir más β-caroteno en la piel

para producir color. Sin embargo, el análisis detallado en cuanto a qué partes del color se vieron afectadas refleja que los cambios asociados al β-caroteno no fueron mediados por el β-caroteno directamente sino indirectamente a través de los iridióforos. Estos resultados evidencian que es necesario un completo conocimiento de las especies de estudio y de los componentes que forman su coloración para poder concluir de forma precisa qué procesos fisiológicos están detrás de los cambios de color. A día de hoy, es muy común el uso de variables que resumen el tono, la saturación y el brillo del espectro completo y que, por lo tanto, están sujetas a la variación de otros componentes del color a parte de los carotenoides (Montgomerie 2006). A la luz del presente estudio, es aconsejable la realización de estudios para comprobar previamente qué componentes están implicados en el color y cómo estos componentes dan lugar a cambios de color y elegir en función de estos estudios la variables espectrales que mejor reflejen los procesos fisiológicos subyacentes, lo cual es fundamental para entender la evolución de dichas señales.

El presente estudio aclara la controversia vertida por los estudios, aparentemente contradictorios, realizados en *L. vivipara* (Cote et al. 2008; Vercken et al. 2008), los cuales intentaban dirimir si la coloración en esta especie se trata de un rasgo continuo indicador de la condición (Cote et al. 2008; Cote et al. 2010) o un rasgo discreto, cuya variación es indicativa de distintas estrategias reproductivas (Sinervo et al. 2007; Vercken et al. 2008; Vercken et al. 2010). A partir de los resultados obtenidos, se concluye que dicha controversia en realidad nace del hecho de que la coloración en *L. vivipara* es un rasgo compuesto, cuya variación no se limita a un solo compuesto si no que surge de la combinación de los carotenoides, que dan lugar a variación discreta y, por tanto, a los morfotipos de color, y a los iridióforos, que dan lugar a variación continua en respuesta a las condiciones ambientales experimentadas por los animales. Por lo tanto, el presente estudio pone de manifiesto la complejidad del color como señal formada por varios componentes y con una funcionalidad múltiple desde el punto de vista de las señales animales (Candolin 2003; Grether et al. 2004). La conjunción de varios mensajes en una única señal entraña ventajas para los receptores de dicha señal ya que les permite obtener más información en el mismo tiempo, minimizando posibles costes asociados al atender a una señal (*e.g.*, tiempo de exposición a depredadores) o facilitando la comparación de los múltiples componentes del fenotipo del emisor de la señal (Candolin 2003). El hecho de que el color sea una señal múltiple formada a su vez por múltiples componentes conlleva que puede estar sometido a distintas presiones selectivas. Como se muestra aquí, los morfotipos y por tanto la cantidad de carotenoides en la piel (capítulo V) están sometidos a selección dependiente de frecuencia, cuya principal consecuencia es el mantenimiento de la variabilidad genotípica y fenotípica (capítulo VI) mientras que los iridióforos es más probable que se encuentren bajo selección direccional al estar asociados con la condición de los individuos (capítulo V). Investigar el papel de las coloraciones basadas en carotenoides en lagartijas provee de nuevos puntos de vista acerca de la evolución de este tipo de ornamentos, ya que, como se demuestra en el presente estudio, no se ven sujetos a los mecanismos que se creen generales en aves y peces.

Conclusiones

1- Los pigmentos anaranjados presentes en la piel de *Lacerta vivipara* son de tipo carotenoide y determina en parte los parámetros de reflectancia que definen la coloración ventral de *L. vivipara*. Se descartó la presencia de pteridinas.

2- Los carotenoides están relacionados con la determinación de los morfotipos de color de los machos de *L. vivipara*. El polimorfismo de color en machos de *L. vivipara* determina su éxito reproductivo así como su supervivencia durante la juventud. Dicho polimorfismo actúa como una señal que refleja distintas estrategias y que dirige la selección. Distintos mecanismos de selección dependiente de frecuencia son responsables de mantener esos polimorfismos al desencadenar ciclos de piedra-papel-tijera.

4- El color de *Lacerta vivipara* no depende de la cantidad de carotenoides ingeridos con la dieta a pesar de que ésta determine sustancialmente la cantidad de carotenoides en sangre, tejidos de reserva y piel.

5- La ausencia de efectos tras suplementar la dieta con carotenoides así como la relación encontrada entre la cantidad de carotenoides en la piel y los morfotipos apoyan un mayor control genético de la coloración basada en carotenoides, lo que contrasta con estudios en aves y peces donde se muestra un mayor peso del componente ambiental.

6- Los iridióforos, en contra, son un componente plástico y que refleja el ambiente y los posibles cambios en la condición de los individuos. Por lo tanto, en contra de la creencia general, los carotenoides, aún presentes en el integumento, no necesariamente son los responsables de indicar la condición de los individuos.

7- El color en esta especie es una señal que puede trasmitir múltiples mensajes a través de los distintos componentes que la forman. Los carotenoides señalan la estrategia vital (el morfotipo) y los iridióforos la condición de los individuos.

8- El presente estudio evidencia que la investigación del papel de los carotenoides en una especie de lagartija y, por tanto, en un modelo animal distinto a los previamente estudiados, cuestiona la generalidad de los paradigmas previamente establecidos en relación a los ornamentos basados en carotenoides. Sugiere que la amplia representación de este tipo de ornamentos en el mundo animal no necesariamente se debe a la predisposición de los carotenoides a dar lugar a señales dependientes de la condición y, por lo tanto, que diferentes mecanismos evolutivos son responsables de la amplia representación de este tipo de ornamentos en el mundo animal.

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